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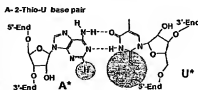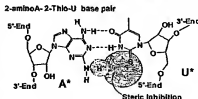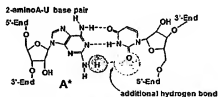
(57) Abstract: Therapeutic sRNA agents and methods of making and using are enclosed.

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iRNA CONJUGATES

RELATED APPLICATIONS

The present application claims the benefit of U.S. Provisional Application
No. 60/460,783, filed April 3, 2003; U.S. Provisional Application No. 60/503,414, filed
September 15, 2003; U.S. Provisional Application No. 60/462,894, filed April 14, 2003; U.S.
Provisional Application No. 60/465,665, filed April 25, 2003; U.S. Provisional Application No.
60/463,772, filed April 17, 2003; U.S. Provisional Application No. 60/465,802, filed April 25,
2003; U.S. Provisional Application No. 60/493,986, filed August 8, 2003; U.S. Provisional
Application No. 60/494,597, filed August 11, 2003; U.S. Provisional Application No.
60/506,341, filed September 26, 2003; U.S. Provisional Application No. 60/518,453, filed
November 7, 2003; U.S. Provisional Application No. 60/469,612, filed May 9, 2003; U.S.
Provisional Application No. 60/510,246, filed October 9, 2003; U.S. Provisional Application
No. 60/510,318, filed October 10, 2003; and International Application No. PCT/US04/07070,
filed March 8, 2004. The contents of these applications are hereby incorporated by reference in
their entirety.

TECHNICAL FIELD

The invention relates to RNAi and related methods, e.g., methods of making and using
iRNA agents. It includes methods and compositions for silencing genes expressed in the kidney,
and methods and compositions for directing iRNA agents to the kidney or to sites other than the
kidney.

BACKGROUND

RNA interference or "RNAi" is a term initially coined by Fire and co-workers to describe
the observation that double-stranded RNA (dsRNA) can block gene expression when it is
introduced into worms (Fire *et al.* (1998) *Nature* 391, 806-811). Short dsRNA directs gene-
specific, post-transcriptional silencing in many organisms, including vertebrates, and has
provided a new tool for studying gene function. RNAi may involve mRNA degradation.

Based on a 1996 estimate from the National Center for Health Statistics, kidney diseases
(including infections, kidney stones, cancer, and a missing kidney) affect 2.55 million U.S.
citizens (Current Estimates from the National Health Interview Survey, 1996). Roughly 400,000

people suffered from end-stage renal disease in 1999. Of these, 150,000 cases resulted from diabetes, 100,000 from hypertension, and 62,000 from glomerulonephritis (the inflammation of the membrane tissue in the kidney) (U.S.R.D.S. 2001 Annual Data Report, 2001). According to a survey by Business Communications Company, Inc., in 2001, the total diabetes drug market was $11.7 billion.

SUMMARY

The kidney is an important site of gene expression. Aspects of the invention relate to silencing genes expressed in the kidney. Accordingly, the invention includes compositions and methods for delivering iRNA agents to the kidney. The invention also includes compositions and methods for minimizing delivery of iRNA agents to the kidney.

Aspects of the invention relate to compositions and methods for silencing genes expressed in the kidney, *e.g.*, to treat disorders of or related to the kidney. An iRNA agent composition of the invention can be one which has been modified to alter distribution in favor of the kidney. A composition of the invention includes an iRNA agent, *e.g.*, an iRNA agent or sRNA agent described herein.

One aspect of the invention provides a method for treating a human having or at risk for having a disorder of the kidney. The method of treatment includes administering an iRNA agent to the human, wherein the iRNA agent targets a nucleic acid, *e.g.*, an RNA expressed in the kidney. In one embodiment, the human is suffering from a disorder characterized by elevated or otherwise unwanted expression of a nucleic acid, *e.g.*, elevated gene expression levels or elevated RNA levels, in the kidney. The unwanted expression levels can correspond to a gene encoding a chemokine, such as RANTES, MCP1 or osteopontin; or a gene encoding a complement factor or a growth factor (*e.g.*, Transforming growth factor-beta (TGFbeta), Platelet derived growth factor (PDGF), IGF-1, IGF-2 or Vascular endothelial growth factor (VEGF)). In another embodiment, the gene can encode an inflammatory cytokine, such as IL1alpha or TNFalpha; a fibrogenic cytokine; a vasoactive protein, such as angiotensin II or ET1; or a growth factor receptor, such as KDR (VEGF receptor), an epidermal growth factor receptor, or a fibroblast growth factor receptor.

In one embodiment, the human has or is at risk for having renal vascular hypertension, a uretar obstruction, diabetes, diabetic nephropathy, glomerular sclerosis, glomerular nephritis,

systemic lupus erythematosis, HIV-associated nephropathy, renal fibrosis, proteinurea, renal carcinoma, Fanconi's syndrome or Bartter's syndrome.

In another embodiment, an iRNA agent targeting the kidney can be administered to a subject in shock, or the agent can be administered before, during, and/or following a kidney transplant.

In one embodiment, the iRNA agent targets a growth factor, such as TGFbeta, or a growth factor receptor, and the human has or is at risk for having diabetic nephropathy, progressive renal disease, chronic tissue injury, or glomerulosclerosis. In one embodiment, the iRNA agent targets a growth factor, such as TGFbeta, and the human has had or is going to have a kidney transplant, or has been identified as a candidate for a kidney transplant.

In one embodiment, the iRNA agent targets PDGF and the human has had or is going to have a kidney transplant, or has been identified as a candidate for a kidney transplant.

In one embodiment, the iRNA agent targets a vasoconstrictor, such as angiotensin II, or a vasoconstrictor receptor, such as angiotensin receptor I, and the human has or is at risk for having angiotensin II-dependent hypertension or type II diabetes, or the human is in a hyperglycemic state.

In one embodiment, the iRNA agent targets a vasoconstrictor, such as endothelin-1 (ET-1), or an ET-1 receptor, such as ETA or ETB, and the human has or is at risk for having an autosomal-dominant polycystic kidney disease and/or chronic renal disease. For example, the human can have an autosomal-dominant polycystic kidney disease, and in one embodiment, the patient's condition has progressed to a chronic renal disease.

In one embodiment, the iRNA agent targets a transcription factor, such as a ligand-activated transcription factor, *e.g.*, the nuclear hormone receptor peroxisome proliferator-activated receptor (PPAR), and the human has or is at risk for having diabetic nephropathy, a kidney tumor, or glomerulosclerosis. In one embodiment the iRNA agent targets PPAR-alpha, PPAR beta/delta, or PPAR gamma.

In one embodiment, the iRNA agent targets a growth factor receptor, such as an IGF receptor (*e.g.*, IGFR1), the VEGF receptor KDR, an epidermal growth factor receptor, or a fibroblast growth factor receptor, and the human has or is at risk for having a renal cell

carcinoma, diabetic nephropathy, renal hypertrophy, glomerular enlargement, increased urinary albumin excretion, and/or diabetes.

In one embodiment, the iRNA agent targets a costimulatory molecule, e.g., B7-1, B7-2, ICOS, CD40, and/or CD154, and the human has or is at risk for having an autoimmune disease or transplant rejection.

In one embodiment, the iRNA agent targets a chemokine, such as MCP-1, RANTES and/or osteopontin, and the human has or is at risk for having systemic hypertension, renal parenchymal injury, an acute or chronic rejection of a kidney allograft, or chronic hypoxia-induced hypertension.

In one embodiment, the iRNA agent targets a sodium-glucose cotransporter, such as sodium-glucose cotransporter 2 (SGLT2), SGLT3, or SGLT1. In one embodiment, the iRNA agent targets SGLT2 (e.g., Homo sapiens solute carrier family 5, member 2 (SLC5A2)) (see FIG. 6), and, e.g., the human has or is at risk for having a renal disease, e.g., early stage renal disease. In one embodiment, an iRNA agent targets a human SLC5A2 nucleic acid, and has a strand that includes a sequence shown in Table 1.

Table 1. Exemplary siRNA oligonucleotides target SLC5A2 mRNA

Position[1]	Region of SLC5A2 transcript	siRNA Sequence	siRNA GC content
148	Coding region	CCATGTGCAGAACCAACAG (SEQ ID NO:2)	53%
499	Coding region	TCTCCGGAGCTGTATTCAT (SEQ ID NO:3}	47%
859	Coding region	TCCTCGGACTCACAATCGT (SEQ ID NO:4)	53%
1311	Coding region	GTGTTCATCGTGGTAGTGT (SEQ ID NO:5)	47%
1701	Coding region	TTCAGTCTCCGGCATAGCA (SEQ ID NO:6)	53%

[1]Nucleotide position of first nt of the siRNA sequence with respect to SEQ ID NO:1 (see FIG. 6).

One aspect of the invention provides an iRNA agent which targets a complement component, such as complement factor C3, C4, C5 or B. An iRNA that targets a complement component can be desirable, e.g., to inhibit the immune response.

In one embodiment, the iRNA agent is at least 21 nucleotides long, and the duplex region of the iRNA is about 19 nucleotides in length.

In one aspect, the invention provides for a method of delivering an iRNA agent to the kidney of a subject, *e.g.*, a mammalian subject, such as a mouse or a human. In one embodiment, the iRNA agent can be delivered to a cell or cells in the glomerulus of the kidney, *e.g.*, glomerular endothelial cells, glomerular epithelial cells, mesangial cells, and the like; and/or the iRNA agent can be delivered to the proximal tubular cells of the kidney. For example, an iRNA agent can be delivered to the proximal tubular cells of the kidney for treatment of shock, uretar obstruction, diabetes, proteinuria, renal carcinoma, or a tubular defect disease, such as Fanconi or Bartter's syndrome. An iRNA agent directed to the treatment of a renal transplant patient can also be directed to the proximal tubular cells of the kidney. In one embodiment, an iRNA directed to the proximal tubular cells of the kidney will further be delivered to the interstitium and other downstream cells. It is preferable that the iRNA agent silences a target gene at the target site within the kidney.

An iRNA agent delivered to the kidney, *e.g.*, the proximal tubular cells of the kidney, can be an unmodified iRNA agent. In one embodiment, the iRNA agent can be stabilized with phosphodiester linkages. In another embodiment, the 3' end of the sense or antisense strand, or both, of the iRNA agent can be modified by a cationic group, *e.g.*, an alkyl amine (such as an 2'O-alkyl amine), polyamine, cationic peptide, or cationic amino acid. The modification can be an external or terminal cationic residue. In another embodiment, the sense or antisense strand, or both, of the iRNA agent can be modified with a sugar, e.g., a glycoconjugate or alkylglycoside component, *e.g.*, glucose, mannose, 2-deoxy-glucose, or an analog thereof. In another embodiment, the iRNA agent can be conjugated to an enzyme substrate, *e.g.*, a substrate for which the relative enzyme is present in a higher amount, as compared to the enzyme level in other tissues of the body. For example, the iRNA agent can be conjugated to a substrate of γ-glutamyl transferase or n-acetyl-γ-glutamyl transferase.
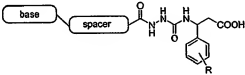
In one embodiment, the iRNA agent can be conjugated to a folic acid or folic acid derivative, *e.g.*, γ-folate, α-folate, 5-methyl tetrahydrofolic acid, a pteridine analog, or an alternative analog thereof.

In one embodiment, the iRNA agent of the invention can be conjugated to a protein that will accumulate in the kidney when administered systematically. For example, the iRNA agent can be conjugated to a lysozyme, cytochrome-c or aprotinin protein. In one embodiment, the iRNA agent can be conjugated to a lysine residue of the protein.

In one embodiment, an iRNA agent targeted to the kidney can be conjugated to a low molecular weight polyethylene glycol (PEG) molecule, or guanidium group, and in another embodiment, the iRNA agent can be conjugated to an RGD peptide, peptide analog, or peptide mimetic or derivative thereof. An iRNA conjugated to an RGD peptide, peptide analog, or peptide mimetic can bind to an αvβ3 integrin. Other exemplary integrin inhibitors are shown in Tables 2 and 3 below.

Table 2.   Synthetic integrin inhibitors described in Goodman *et al.*, *J. Med. Chem.* 45:1045-1051, 2002.

Table 3. Synthetic integrin inhibitors described by Sulyok *et al.*, *J. Med. Chem.* 44:1938-1950, 2001.

	R	base	spacer
	(Carboxamide)[a]	guanidine	m-C_6H_4-
	none	guanidine	ω-C_4H_8-
	none	guanidine	m-C_6H_4-
	4-F	guanidine	m-C_6H_4-
	4-Cl	guanidine	m-C_6H_4-
	4-Br	guanidine	m-C_6H_4-
	4-OCH_3	guanidine	m-C_6H_4-
	4-OCF_3	guanidine	guanidine
	(1-naphthyl)[a]	guanidine	m-C_6H_4-
	3-Cl,5-Cl	guanidine	m-C_6H_4-
	(H)[a]	2-NH_2-pyridine	ω-C_4H_8-
	none	2-NH_2-pyridine	ω-C_4H_8-
	4-F	2-NH_2-pyridine	ω-C_4H_8-
	4-Cl	2-NH_2-pyridine	ω-C_4H_8-
	4-Br	2-NH_2-pyridine	ω-C_4H_8-
	4-OCH_3	2-NH_2-pyridine	ω-C_4H_8-

	4-OCF$_3$	2-NH$_2$-pyridine	ω-C$_4$H$_8$-
	(1-naphthyl)[a]	2-NH$_2$-pyridine	ω-C$_4$H$_8$-
	3-Cl,5-Cl	2-NH$_2$-pyridine	ω-C$_4$H$_8$-

[a] Instead of substituted phenyl ring

Ref: Sulyok, G. A. G.; Gibson, C.; Goodman, S. L.; Hölzemann, G.; Wiesner, M.; Kessler H. *J. Med. Chem.* **2001**, *44*, 1938-1950

In a preferred embodiment, at least 30%, 40%, 50%, 60%, 70%, 80%, 90% or more of the iRNA agent administered to the subject is successfully targeted to the the kidney. In a preferred embodiment between 30-90%, 40-80% or 50-70% 50-80%, or 50-90% of the iRNA agent administered to the subject is successfully targeted to the the kidney.

In any of the embodiments described above, the iRNA agent/conjugate can have additional modifications, such as a stabilizing modification. For example, a linker molecule can tether a protein, PEG or RGD peptide to the iRNA agent. Exemplary linkers are described *infra*, and can include amino linkers (e.g., aminooxy linkers), thiol linkers, carboxyl linkers, aldehyde linkers, haloacetyl linkers, and the like.

In another aspect, the invention features an iRNA conjugate. The conjugate includes an iRNA agent coupled to, e.g., linked to, a ligand or therapeutic agent. The iRNA agent is optionally coupled to the ligand or therapeutic agent by a linker (e.g., a peptide linker or other linker described herein). The ligand can function to, e.g., affect the distribution of the iRNA agent in the body and/or to target the iRNA agent to a particular tissue or cell.

The ligand can be placed at an end of the iRNA agent, preferably at the 3'end of an RNA strand of the iRNA agent. The ligand can also be placed at the 5'end, or within the middle of the iRNA agent. In some embodiments, more than one ligand can be coupled to the iRNA agent. For example, a ligand can be coupled to the 3' end of each of two strands of an iRNA agent; a ligand can be coupled to an end, e.g., a 3' end and to the middle of a strand of an iRNA agent; a ligand can be coupled to the 3' end and the 5' of one or both of two strands of an iRNA agent.

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In one embodiment, the ligand is a lipid or lipid-based molecule. Such a lipid or lipid-based molecule preferably binds a serum protein, e.g., human serum albumin (HSA). An HSA binding ligand allows for distribution of the conjugate to a target tissue, e.g., a non-kidney target tissue of the body. For example, the target tissue can be the liver, including, but not limited to parenchymal cells of the liver. Other molecules that can bind HSA can also be used as ligands. For example, neproxin or aspirin can be used. A lipid or lipid-based ligand can (a) increase resistance to degradation of the conjugate, (b) increase targeting or transport into a target cell or cell membrane, and/or (c) can be used to adjust binding to a serum protein, e.g., HSA.

A lipid based ligand can be used to modulate, e.g., control the binding of the conjugate to a target tissue. For example, a lipid or lipid-based ligand that binds to HSA more strongly will be less likely to be targeted to the kidney and therefore less likey to be cleared from the body. A lipid or lipid-based ligand that binds to HSA less strongly can be used to target the conjugate to the kidney.

In a preferred embodiment, the lipid based ligand binds HSA. Preferably, it binds HSA with a sufficient affinity such that the conjugate will be preferably distributed to a non-kidney tissue. However, it is preferred that the affinity not be so strong that the HSA-ligand binding cannot be reversed.

In another preferred embodiment, the lipid based ligand binds HSA weakly or not at all, such that the conjugate will be preferably distributed to the kidney. Other moieties that target to kidney cells can also be used in place of or in addition to the lipid based ligand.

In a preferred embodiment, the lipid or lipid based ligand is a phosphorothioate. In this embodiment, it is preferred that the number of sulfurs on the phosphorothioate not be so prevalent that they interfere with binding to a serum protein, e.g., HSA.

In another embodiment, the ligand is a peptide or peptoid. Peptoids, in particular ampiphathic species, such as Antennapedia or tat, are preferred.

In another embodiment, the ligand is a polyethylene glycol (PEG) or derivatives thereof. A PEG can, e.g., allow the agent to be kept in circulation. A PEG is intrinsically amphipathic, and can promote stability, particularly if coupled at the 3'end of the iRNA agent.

In another embodiment, the ligand is a charged group or moiety, e.g., a polyamine or cationic group or moiety. This type of linker moiety, e.g., because of its charge, e.g., its negative

charge, can help overcome the resistance of entry of the iRNA agent into a cell. Preferably, these are conjugated at the 3' end, but they can also be at the 5' end or within the middle of the iRNA molecule. Exemplary polyamines include polyarginine, polylysine, polyhistidine, polypreprozine, or polymorpholinos, polyornithine.

5   In another embodiment, the ligand is a vitamin or other moiety which is taken up by a target cell, e.g., a proliferating cell. These are particularly useful for treating disorders characterized by unwanted cell proliferation, e.g., of the malignant or non-malignant type, e.g., cancer cells. Exemplary vitamins are B vitamin, e.g., folic acid, B12, riboflavin, biotin, pyridoxal or other vitamins or nutrients taken up by cancer cells. Also included are HSA and
10   low density lipoprotein (LDL).

        In another embodiment, the ligand is a cell-permeation agent, preferably a helical cell-permeation agent. Preferably, the agent is amphipathic. An exemplary agent is a peptide such as tat or antennopedia. If the agent is a peptide, it can be modified, including a pepidylmimetic, invertomers, non-peptide or pseudo-peptide linkages, and use of D-amino acids. The helical
15   agent is preferably an alpha-helical agent, which preferably has a lipophilic and a lipophobic phase.

        The ligand or targeting agent can be a targeting agent. The targeting agent can be a sugar, a peptide, e.g., an RGD containing peptide.

        Another useful targeting agent is one that incorporates a sugar, e.g., galactose and/or
20   analogs thereof. These are useful because they target the liver, in particular, the parenchymal cells of the liver. In a preferred embodiment, the targeting moiety includes more than one galactose moiety, preferably two or three. Preferably, the targeting moiety includes 3 galactose moieties, e.g., spaced about 15 angstroms from each other. The targeting moiety can be lactose. A lactose is a glucose coupled to a galactose. Preferably, the targeting moiety includes three
25   lactoses. The targeting moiety can also be N-Acetyl-Galactosamine, N-Ac-Glucosamine. A mannose, or mannose-6-phosphate targeting moiety can be used for macrophage targeting.

        Peptides that target markers enriched in proliferating cells can be used. E.g., RGD containing peptides and petomimetics can target cancer cells, in particular cells that exhibit an $\alpha_v\beta_3$ integrin. Thus, one could use RGD peptides, cyclic peptides containing RGD, RGD
30   peptides that include D-amino acids, as well as synthetic RGD mimics. In addition to RGD, one can use other moieties that target the α_v-β_3 integrin ligand. Generally, such ligands can be used

to control proliferating cells and angiogeneis. Preferred conjugates of this type include an iRNA agent that targets PECAM-1, VEGF, or other cancer gene, e.g., a cancer gene described herein.

In one embodiment, an iRNA agent is linked, e.g., directly linked, e.g., covalently, or non-covalentlylinked, to the targeting agent, e.g., a targeting agent decribed herein. This is referred to as a "conjugation" approach. In another embodiment, the targeting agent (e.g., the same targting agent) is simply mixed with the iRNA agent. This is referred to as a "complexing" approach. In a complexing approach, the iRNA agent can be mixed with, e.g., a cationic molecule, e.g., a cationic lipid, e.g., with or without a targting group, e.g., with or without a sugar or an RGD construct described herein. In some embodiments, the iRNA agent is mixed with a polymer-based system, e.g., with or without a targeting group. In other embodiments, the iRNA agent is mixed with a nanoparticle.

The iRNA conjugates described herein can include a targeting agent that targets the iRNA agent to a desired target cell or tissue. The target cell or tissue can be a cancer cell, a cell of the vasculature, e.g, tumor vasculature, an angiogenic cell, e.g., a tumor angiogenic cell, or an endosome. A preferred target is the kidney. In another embodiment, the liver e.g., the parenchymal cells of the liver, is a preferred target.

The methods and compositions of the invention, e.g., the conjugates described herein, can be used with any of the iRNA agents described herein. In addition, the methods and compositions of the invention can be used for the treatment of any disease or disorder described herein, and for the treatment of any subject, e.g., any animal, any mammal, such as any human.

The methods and compositions of the invention, e.g., the conjugates described herein, can be used with any dosage and/or formulation described herein, as well as with any route of administration described herein.

As used herein, "conjugated" means two entities are associated, e.g., with sufficient affinity that the therapeutic benefit of the association between the two entities is realized. Conjugated can include covalent or noncovalent bonding as well as other forms of association, such as entrapment, e.g., of one entity on or within the other, or of either or both entities on or within a third entity, such as a micelle. Particularly preferred forms of conjugation are by covalent bonding, e.g., those described herein. An entity can be conjugated to an iRNA agent, e.g., at the 3' or 5' terminus of either strand or internally. It is preferred that an entity is

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conjugated to the iRNA agent in such a way as to preserve the ability of the antisense strand to mediate silencing.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. The materials, methods, and examples are illustrative only and not intended to be limiting. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, useful methods and materials are described below. Other features and advantages of the invention will be apparent from the accompanying drawings and description, and from the claims. The contents of all references, pending patent applications and published patents, cited throughout this application are hereby expressly incorporated by reference. In case of conflict, the present specification, including definitions, will control.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a structural representation of base pairing in psuedocomplementary siRNA[2].

FIG. 2 is a schematic representation of dual targeting siRNAs designed to target the HCV genome.

FIG. 3 is a schematic representation of psuedocomplementary, bifunctional siRNAs designed to target the HCV genome.

FIG. 4 is a general synthetic scheme for incorporation of RRMS monomers into an oligonucleotide.

FIG. 5 is a table of representative RRMS carriers. Panel 1 shows pyrroline-based RRMSs; panel 2 shows 3-hydroxyproline-based RRMSs; panel 3 shows piperidine-based RRMSs; panel 4 shows morpholine and piperazine-based RRMSs; and panel 5 shows decalin-based RRMSs. R1 is succinate or phosphoramidate and R2 is H or a conjugate ligand.

FIG. 6 is the nucleotide sequence of *Homo sapiens* solute carrier family 5 (sodium/glucose cotransporter), member 2 (SLC5A2) mRNA (SEQ ID NO:1). The 5′ and 3′ UTRs are indicated by italics. Exemplary siRNA target regions are underlined.

FIG. 7A is a graph depicting the results of quantitative RT-PCR experiments. Class I iRNAs were injected into mice, and then liver and kidney ApoM RNA levels were measured by quantitative RT-PCR. "C1" and "C2" represents ApoM RNA levels in control mice.

5   FIG. 7B shows gels denoting the results of quantitative RT-PCR experiments. Class I iRNAs were injected into mice, and then liver and kidney ApoM RNA levels were measured by quantitative RT-PCR. "C1" and "C2" represents ApoM RNA levels in control mice.

FIG. 8 is a graph showing the results of quantitative RT-PCR experiments that measured ApoM RNA levels in HepG2 tissue culture cells following cotransfection with a plasmid expressing exogenous ApoM RNA under a CMV promoter and a class I iRNA (1, 2, 3, or 4).
10 "C" represents a ApoM RNA levels in control HepG2 tissue culture cells.

FIG. 9A is a graph depicting the results of quantitative RT-PCR experiments. Class II iRNAs (11, 13, 15, and 17) were injected separately into mice. Liver and kidney ApoM RNA levels were then measured by quantitative RT-PCR. "C" represents ApoM RNA levels in control mice (mice not injected with class II iRNAs).

15 FIG. 9B shows gels denoting the results of quantitative RT-PCR experiments. Class II iRNAs were injected into mice, and then liver and kidney ApoM RNA levels were measured by quantitative RT-PCR. "C" represents ApoM RNA levels in control mice (mice not injected with class II iRNAs).

FIG. 10A is a graph showing the levels of serum ApoM levels in mice following injection
20 with class II RNAi's (11, 13, 15, or 17) containing phosphorothioates. "C" represents serum ApoM levels in control mice (mice not injected with class II iRNAs).

FIG. 10B is a Western blot showing the levels of serum ApoM levels in mice following injection with class II RNAi containing phosphorothioates. "C" represents serum ApoM levels in control mice (mice not injected with class II iRNAs).

25 FIG. 11A is a graph showing the levels of serum ApoM levels in mice following injection with Class III RNAi molecules (19, 21, or 23). "C" represents serum ApoM levels in control mice (mice not injected with class III iRNAs).

FIG. 11B is a Western blot showing the levels of serum ApoM levels in mice following injection with Class III RNAi molecules. "C" represents serum ApoM levels in control mice (mice not injected with class III iRNAs).

FIG. 12A is a graph showing the levels of serum ApoM levels in mice following injections with varying concentrations ("μg") of RNAi. The effect of preincubating the RNAi with lipofectamine ("Lipo") was also tested in these experiments.

FIG. 12B is a Western blot showing the levels of serum ApoM levels in mice following injections with varying concentrations ("μg") of RNAi. The effect of preincubating the RNAi with lipofectamine ("Lipo") was also tested in these experiments.

FIG. 13 depicts a sugar moiety useful for conjugation to an iRNA agent (depicted by "siRNA"). "X" can be S, NH, NR, or O. "R" can be an alkyl group.

DETAILED DESCRIPTION

Double-stranded (dsRNA) directs the sequence-specific silencing of mRNA through a process known as RNA interference (RNAi). The process occurs in a wide variety of organisms, including mammals and other vertebrates.

It has been demonstrated that 21-23 nt fragments of dsRNA are sequence-specific mediators of RNA silencing, e.g., by causing RNA degradation. While not wishing to be bound by theory, it may be that a molecular signal, which may be merely the specific length of the fragments, present in these 21-23 nt fragments recruits cellular factors that mediate RNAi. Described herein are methods for preparing and administering these 21-23 nt fragments, and other iRNAs agents, and their use for specifically inactivating gene function. The use of iRNAs agents (or recombinantly produced or chemically synthesized oligonucleotides of the same or similar nature) enables the targeting of specific mRNAs for silencing in mammalian cells. In addition, longer dsRNA agent fragments can also be used, e.g., as described below.

Although, in mammalian cells, long dsRNAs can induce the interferon response which is frequently deleterious, sRNAs do not trigger the interferon response, at least not to an extent that is deleterious to the cell and host. In particular, the length of the iRNA agent strands in an sRNA agent can be less than 31, 30, 28, 25, or 23 nt, e.g., sufficiently short to avoid inducing a deleterious interferon response. Thus, the administration of a composition of sRNA agent (e.g., formulated as described herein) to a mammalian cell can be used to silence expression of a target

gene while circumventing the interferon response. Further, use of a discrete species of iRNA agent can be used to selectively target one allele of a target gene, *e.g.*, in a subject heterozygous for the allele.

Moreover, in one embodiment, a mammalian cell is treated with an iRNA agent that
5 disrupts a component of the interferon response, *e.g.*, double stranded RNA (dsRNA)-activated protein kinase PKR. Such a cell can be treated with a second iRNA agent that includes a sequence complementary to a target RNA and that has a length that might otherwise trigger the interferon response.

In a typical embodiment, the subject is a mammal such as a cow, horse, mouse, rat, dog,
10 pig, goat, or a primate. The subject can be a dairy mammal (*e.g.*, a cow, or goat) or other farmed animal (*e.g.*, a chicken, turkey, sheep, pig, fish, shrimp). In a much preferred embodiment, the subject is a human, *e.g.*, a normal individual or an individual that has, is diagnosed with, or is predicted to have a disease or disorder.

Further, because iRNA agent mediated silencing persists for several days after
15 administering the iRNA agent composition, in many instances, it is possible to administer the composition with a frequency of less than once per day, or, for some instances, only once for the entire therapeutic regimen. For example, treatment of some cancer cells may be mediated by a single bolus administration, whereas a chronic viral infection may require regular administration, *e.g.*, once per week or once per month.

20 A number of exemplary routes of delivery are described that can be used to administer an iRNA agent to a subject. In addition, the iRNA agent can be formulated according to an exemplary method described herein.

Targeting of iRNA Agents to the Kidney

The delivery of iRNA agents to the kidney can be preferable, such as for the treatment of
25 diseases of the kidney. An iRNA agent that is targeted to the kidney can be delivered to a particular cell type of the kidney, and further to a particular gene target of the kidney. For example, an iRNA agent of the invention can be directed to cells of the glomerulus (*e.g.*, glomerular endothelial cells, glomerular epithelial cells, or mesangial cells), or to proximal tubular cells of the kidney. An iRNA agent delivered to the proximal tubular cells of the kidney
30 can be further distributed to the interstitium and other downstream cells.

The invention includes a variety of methods for the distribution of iRNA agents to the kidney. The distribution of iRNAs can be targeted to the kidney by the incorporation of a phosphodiester backbone. For example, an iRNA agent targeted to the kidney can have at least about 10, 15, 20, 25, or 30 phosphodiester linkages or more incorporated into the backbone. The

5   phosphodiester backbone is particularly efficient at delivery of an iRNA agent to the proximal tubular cells of the kidney. Delivery of the iRNA agent can be enhanced by the attachment of a modification, such as a cationic group, to at least one end of, or internally on, the sense or antisense strand of the iRNA molecule, or both. Preferred points of attachment are, for example, to the 5' end of the sense strand, the 3' end of the sense strand or the 3' end of the antisense

10   strand. Exemplary cationic groups include alkyl amines, polyamines, cationic peptides, and cationic amino acids (e.g., arginine, lysine, or ornithine). In one alternative, the cationic group modification can be attached to the 3' end of an iRNA agent. In another alternative, the cationic group is conjugated internally on the iRNA agent, e.g., by any of the methods described herein. Lateral conjugation is preferred to be on the sense strand. Exemplary cationic modification are

15   shown below.

2'- Cationic Modifications
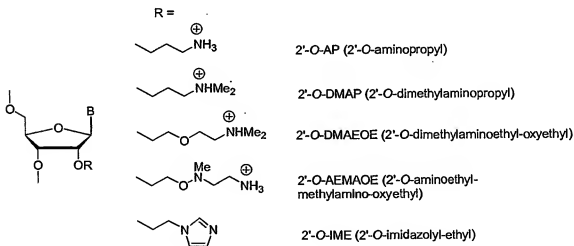
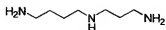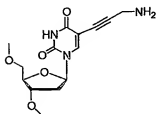Cationic 2'-modifications in the protonated form at the physiological pH

Cationic groups
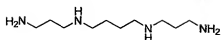
Spermine

Spermidine

5-(3-Amino-1-propynyl)-2'-deoxyuridine
residue
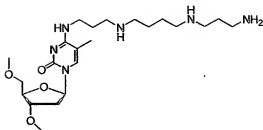
5-Methyl-2-oxo-4-spermine-1-(2-deoxyribose)pyrimidine
residue
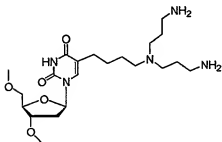
5-[4-[N,N-bis-(3-aminopropyl)amino]butyl]-
2'-deoxyuridine residue
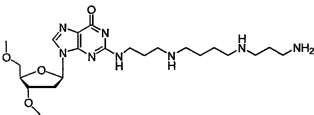
2-Spermine-2'-deoxyinosine residue
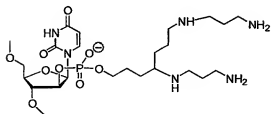
Ara-Uridine-2'-(sperminepropyl)phosphate residue

Cationic base modifications
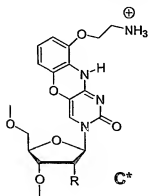
G clamp

An iRNA agent can be targeted to the kidney by conjugation of a sugar moiety to the
iRNA agent, as shown in FIG. 13 for example. Exemplary sugar molecules include glucose,
mannose, and 2-deoxy-glucose, and analogs of each. These particular sugars and others have
specific receptors in the kidney, and are therefore useful for delivery of an iRNA agent to the
kidney. The sugar moiety can include a hydrophobic group, e.g., an alkyl group, attached to the
anomeric carbon. For example, the hydrophobic group can be attached via a carbon, sulfur,
oxygen, or nitrogen atom, e.g., an amino group. The sugar moiety can be attached to the iRNA
agent, e.g., by a carbamate linker, or by any of the methods described herein.

An iRNA agent can be delivered to the kidney through the exploitation of a kidney-
selective enzyme, e.g., an enzyme that expressed and or active primarily in the kidney. An iRNA
agent can be conjugated with a substrate for an enzyme that is found in the kidney, preferably an
enzyme that is enriched in or is localized to the kidney. For example, the γ-glutamyl
transpeptidase, γ-glutamyl transferase and n-acetal-γ-glutamyl enzymes are more abundant in the
proximal tubular cells of the kidney than in some other tissue types. Therefore, an iRNA agent
conjugated to glutathione or a γ-glutamyl amino acid will be transported to and concentrated in
the kidney proximal tubular cells where the enzymes of the γ-glutamyl cycle are concentrated.

The kidney also contains a high-affinity folate binding protein (FBP), also concentrated
in the proximal tubular cells (Christensen *et al., Int. Rev. Cytol.* 180:237-284, 1998). Therefore,
an iRNA agent can be targeted to the kidney, by conjugation of the iRNA agent to a folic acid
molecule. This delivery method has been shown to successfully deliver antisense RNA messages

to the kidney. Exemplary folic acid conjugates include, γ-folate, α-folate, 5-methyl tetrahydrofolic acid, pteridine analogs and other folic acid analogs.

Certain low molecular weight proteins (e.g., lysozyme, cytochrome C or aprotinin) when administered systematically, accumulate rapidly in the kidney, where they concentrate in the proximal tubular cells through a reabsorption mechanism. An iRNA agent can be conjugated to one of these proteins, e.g., via a lysine residue of the protein. For example, a linker moiety (see infra) can tether the iRNA agent to the lysine residue. An iRNA-protein conjugate will, in some cases, be more resistant to nucleases than an iRNA agent alone. After delivery of the iRNA-protein conjugate to the proximal tubules, proteases of the lysosome can optionally liberate the iRNA agent from the protein conjugate, thereby freeing the iRNA agent to anneal to its target nucleic acid. In one embodiment, a fusogenic component of the complex, e.g., a fusogenic agent conjugated to the iRNA-protein complex, can facilitate the release of the iRNA agent from a lysosome or endosome.

An iRNA agent can be targeted to the kidney using a method that relies upon extensive hydration which can be effected, e.g., by conjugation to a moiety, e.g., a polymer, e.g., a polyethylene glycol (PEG). For example, an iRNA agent can be fused to a water soluble polymer, e.g., a small-molecular weight PEG molecule, which will transport siRNAs to the kidney. The PEG molecule can have a molecular weight of about 500, 600, 900, 1000, 2000, 10000, 25000, 50000 or 100,000. Preferably, the PEG has a molecular weight of between about, 500 and 100000, 2000 and 50000. More preferebly, the PEG has a molecular weight between about 5000 and 40000.

An iRNA of the invention can be targeted to the mesangial cells of the kidney by conjugation of a peptide containing one or more Arg-Gly-Asp (RGD) motifs. The RGD motif interacts with integrins of the mesangial cells of the kidney. For example, an iRNA-RGD conjugate can bind to an alphaV-beta3, alpha8, alpha5, or alpha5-beta1 integrin, or to other integrins concentrated in the mesangial cells of the kidney. In other alternatives, an iRNA agent of the invention can be conjugated to an RGD analog or RGD mimic.

An iRNA agent can be used to treat a human having or at risk for having a disease or disorder associated with the kidney. For example, an iRNA of the invention can be administered to treat renal vascular hypertension, diabetes (or a symptom of diabetes such as diabetic nephropathy), glomerular sclerosis, glomerular nephritis, systemic lupus erythematosus, HIV-

associated nephropathy, renal carcinoma, renal fibrosis, or inflammatory diseases that may
eventually lead to necessary renal transplantation.

There are a variety of exemplary gene targets useful for the treatment of kidney-related
disorders. For example, an iRNA agent of the invention can target a nucleic acid encoding a
cytokine or a growth factor (e.g., TGFbeta, PDGF, IGF-1, IGF-2, or VEGF). An iRNA agent can
target the growth factor TGFbeta, for example, which has been shown to contribute to
progressive diabetic nephropathy (Reeves and Andreoli, *Proc. Natl. Acad. Sci.* 97:7667-7669,
2000). TGFbeta has also emerged as a predominant mediator of extracellular matrix production
and deposition in progressive renal disease and in other forms of chronic tissue injury (Cheng
and Grande, *Exp. Biol. Med.* 227:943-956, 2002). Inhibition of TGFbeta activity can prevent
renal insufficiency and glomerusclerosis, such as in patients with type II diabetes. In another
example, an iRNA of the invention can target PDGF. Chronic allograft nephropathy is the
primary reason for late allograft loss in kidney transplantation, and inhibition of PDGF can be
administered to transplant patients as a method to prevent graft rejection (see, for example,
Savikko *et al., Transplantation* 27:1147-1153, 2003).

An iRNA agent can target a vasoconstrictor or a vasoconstrictor receptor. For example,
an iRNA agent can be used to target angiotensin II or the AGT1 receptor. Inhibitors of either of
these genes or gene products can be administered for the treatment of angiotensin II-dependent
hypertension. In addition, inhibitors of the angiotensin receptor can slow the progression of
nephropathy, such as in patients with type II diabetes (Lewis, *Am. J. Hypertens.* 15: 123S-128S,
2002). An iRNA agent that targets angiotensin II or the AGT1 receptor can be administered to
patients in a hyperglycemic state to increase renal plasma flow (Hollenberg, *Am. J. Hypertens.*
14:237S-241S, 2001). An iRNA agent can target the vasoconstrictor ET-1, or one or more of its
receptors, ETA or ETB. An iRNA agent that targets ET-1 or an ET-1 receptor can be
administered to a patient with autosomal-dominant polycystic kidney disease. Treatment of the
disease can begin or continue after a patient has developed chronic renal disease.

iRNA agents which silence genes disclosed herein are preferably modified to favor
distribution to the kidney, but will in some cases have sufficient efficacy do not require
modification.

An iRNA agent directed to the kidney can target a ligand-activated transcription factor,
such as a nuclear hormone receptor, e.g., a peroxisome proliferator-activated receptor (PPAR).
Three isoforms of PPAR (alpha, beta/delta, and gamma) are differentially expressed in the

kidney, and any of the isoforms (or two or all three) can be a target of an iRNA agent(s) administered for the treatment of a renal disease, e.g., glomerulosclerosis, diabetic nephropathy, or kidney tumors (Guan, *Minerva Urol. Nefrol.* 54:65-79, 2002).

An iRNA agent can target a growth hormone receptor for the treatment of a kidney disorder, such as renal cell carcinoma (RCC). Exemplary hormone receptor targets for the treatment, e.g., of RCC, include but are not limited to KDR, an EGF receptor, an FGF receptor, and an IGF receptor (*e.g.*, IGFR1 or IGFR2). Targeting of any of these growth hormone receptors can also be useful as a treatment against diabetes.

An iRNA agent can be used to target a component of the immune system, such as a costimulatory molecule, such as B7-1, B7-2, ICOS, CD40, CD154, and the like. Disruption of the natural costimulatory interaction can be effective in the prevention and treatment of autoimmune disease and transplant rejection (Biancone *et al., Nephrol.* 15:7-16, 2002).

An iRNA agent can be used to target a chemokine, such as RANTES, MCP-1, or osteopontin. An iRNA agent that targets RANTES or MCP-1, for example, can inhibit monocyte and macrophage recruitment into the kidney, such as may occur in cases of systemic hypertension, and consequently, administration of these iRNA agents can inhibit or prevent renal parenchymal injury. An iRNA agent that targets MCP-1 can be administered to inhibit the acute and chronic rejection of a kidney allograft (Lazzeri, *G. Ital. Nefrol.* 19:641-649, 2002). An iRNA agent that targets osteopontin can be administered to treat hypertension.

An iRNA agent can be used to target a complement component, such as complement factor C3, C4, C5 or B. An iRNA agent directed against a complement factor can be administered to a patient to treat or prevent nephropathy and/or glomerulonephritis (see Hanafusa *et al., Nephrol. Dial. Transplant* 17, Suppl 9:34-36, 2002; and Welch, *Nephron* 88:199-204, 2001).

A patient who has had or is identified as being a candidate for a kidney transplant can be treated with an iRNA that targets the kidney. A kidney transplant can refer to an allo- or xenotransplant, and an allotransplant can utilize the kidney of an HLA-matched or mismatched donor. An iRNA agent can be administered prior to, concurrent with, or after the transplant. An iRNA agent targeted to the kidney can be combined with other therapeutics, such as broad spectrum immunosuppressors, e.g., cyclosporin A, Tacrolimus (FK506), sirolimus (rapamycin);

anti-T cell antibodies, e.g., OKT3 therapy, anti-CD3, anti-CTLA4, or anti-CD28; or radiation therapy.

iRNA AGENT STRUCTURE

Described herein are isolated iRNA agents, *e.g.*, RNA molecules, (double-stranded; single-stranded) that mediate RNAi. The iRNA agents preferably mediate RNAi with respect to an endogenous gene of a subject or to a gene of a pathogen.

An "RNA agent" as used herein, is an unmodified RNA, modified RNA, or nucleoside surrogate, all of which are defined herein (see, e.g., the section below entitled RNA Agents). While numerous modified RNAs and nucleoside surrogates are described, preferred examples include those which have greater resistance to nuclease degradation than do unmodified RNAs. Preferred examples include those which have a 2' sugar modification, a modification in a single strand overhang, preferably a 3' single strand overhang, or, particularly if single stranded, a 5' modification which includes one or more phosphate groups or one or more analogs of a phosphate group.

An "iRNA agent" as used herein, is an RNA agent which can, or which can be cleaved into an RNA agent which can, down regulate the expression of a target gene, preferably an endogenous or pathogen target RNA. While not wishing to be bound by theory, an iRNA agent may act by one or more of a number of mechanisms, including post-transcriptional cleavage of a target mRNA sometimes referred to in the art as RNAi, or pre-transcriptional or pre-translational mechanisms. An iRNA agent can include a single strand or can include more than one strands, *e.g.*, it can be a double stranded iRNA agent. If the iRNA agent is a single strand it is particularly preferred that it include a 5' modification which includes one or more phosphate groups or one or more analogs of a phosphate group.

The iRNA agent should include a region of sufficient homology to the target gene, and be of sufficient length in terms of nucleotides, such that the iRNA agent, or a fragment thereof, can mediate down regulation of the target gene. (For ease of exposition the term nucleotide or ribonucleotide is sometimes used herein in reference to one or more monomeric subunits of an RNA agent. It will be understood herein that the usage of the term "ribonucleotide" or "nucleotide", herein can, in the case of a modified RNA or nucleotide surrogate, also refer to a modified nucleotide, or surrogate replacement moiety at one or more positions.) Thus, the iRNA agent is or includes a region which is at least partially, and in some embodiments fully,

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complementary to the target RNA. It is not necessary that there be perfect complementarity between the iRNA agent and the target, but the correspondence must be sufficient to enable the iRNA agent, or a cleavage product thereof, to direct sequence specific silencing, e.g., by RNAi cleavage of the target RNA, e.g., mRNA.

5   Complementarity, or degree of homology with the target strand, is most critical in the antisense strand. While perfect complementarity, particularly in the antisense strand, is often desired some embodiments can include, particularly in the antisense strand, one or more but preferably 6, 5, 4, 3, 2, or fewer mismatches (with respect to the target RNA). The mismatches, particularly in the antisense strand, are most tolerated in the terminal regions and if present are

10   preferably in a terminal region or regions, e.g., within 6, 5, 4, or 3 nucleotides of the 5' and/or 3' terminus. The sense strand need only be sufficiently complementary with the antisense strand to maintain the over all double strand character of the molecule.

As discussed elsewhere herein, an iRNA agent will often be modified or include nucleoside surrogates in addition to the RRMS. Single stranded regions of an iRNA agent will

15   often be modified or include nucleoside surrogates, e.g., the unpaired region or regions of a hairpin structure, e.g., a region which links two complementary regions, can have modifications or nucleoside surrogates. Modification to stabilize one or more 3'- or 5'-terminus of an iRNA agent, e.g., against exonucleases, or to favor the antisense sRNA agent to enter into RISC are also favored. Modifications can include C3 (or C6, C7, C12) amino linkers, thiol linkers,

20   carboxyl linkers, non-nucleotidic spacers (C3, C6, C9, C12, abasic, triethylene glycol, hexaethylene glycol), special biotin or fluorescein reagents that come as phosphoramidites and that have another DMT-protected hydroxyl group, allowing multiple couplings during RNA synthesis.

iRNA agents include: molecules that are long enough to trigger the interferon response

25   (which can be cleaved by Dicer (Bernstein et al. 2001. Nature, 409:363-366) and enter a RISC (RNAi-induced silencing complex)); and, molecules which are sufficiently short that they do not trigger the interferon response (which molecules can also be cleaved by Dicer and/or enter a RISC), e.g., molecules which are of a size which allows entry into a RISC, e.g., molecules which resemble Dicer-cleavage products. Molecules that are short enough that they do not trigger an

30   interferon response are termed sRNA agents or shorter iRNA agents herein. "sRNA agent or shorter iRNA agent" as used herein, refers to an iRNA agent, e.g., a double stranded RNA agent or single strand agent, that is sufficiently short that it does not induce a deleterious interferon

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response in a human cell, *e.g.*, it has a duplexed region of less than 60 but preferably less than 50, 40, or 30 nucleotide pairs. The sRNA agent, or a cleavage product thereof, can down regulate a target gene, *e.g.*, by inducing RNAi with respect to a target RNA, preferably an endogenous or pathogen target RNA.

5   Each strand of an sRNA agent can be equal to or less than 30, 25, 24, 23, 22, 21, or 20 nucleotides in length. The strand is preferably at least 19 nucleotides in length. For example, each strand can be between 21 and 25 nucleotides in length. Preferred sRNA agents have a duplex region of 17, 18, 19, 29, 21, 22, 23, 24, or 25 nucleotide pairs, and one or more overhangs, preferably one or two 3' overhangs, of 2-3 nucleotides.

10   In addition to homology to target RNA and the ability to down regulate a target gene, an iRNA agent will preferably have one or more of the following properties:

        (1) it will be of the Formula 1, 2, 3, or 4 set out in the RNA Agent section below;

        (2) if single stranded it will have a 5' modification which includes one or more phosphate groups or one or more analogs of a phosphate group;

15   (3) it will, despite modifications, even to a very large number, or all of the nucleosides, have an antisense strand that can present bases (or modified bases) in the proper three dimensional framework so as to be able to form correct base pairing and form a duplex structure with a homologous target RNA which is sufficient to allow down regulation of the target, *e.g.*, by cleavage of the target RNA;

20   (4) it will, despite modifications, even to a very large number, or all of the nucleosides, still have "RNA-like" properties, *i.e.*, it will possess the overall structural, chemical and physical properties of an RNA molecule, even though not exclusively, or even partly, of ribonucleotide-based content. For example, an iRNA agent can contain, *e.g.*, a sense and/or an antisense strand in which all of the nucleotide sugars contain *e.g.*, 2' fluoro in place of 2' hydroxyl. This

25 deoxyribonucleotide-containing agent can still be expected to exhibit RNA-like properties. While not wishing to be bound by theory, the electronegative fluorine prefers an axial orientation when attached to the C2' position of ribose. This spatial preference of fluorine can, in turn, force the sugars to adopt a C_3-*endo* pucker. This is the same puckering mode as observed in RNA molecules and gives rise to the RNA-characteristic A-family-type helix.

30 Further, since fluorine is a good hydrogen bond acceptor, it can participate in the same hydrogen bonding interactions with water molecules that are known to stabilize RNA structures.

(Generally, it is preferred that a modified moiety at the 2' sugar position will be able to enter into H-bonding which is more characteristic of the OH moiety of a ribonucleotide than the H moiety of a deoxyribonucleotide. A preferred iRNA agent will: exhibit a C_3-*endo* pucker in all, or at least 50, 75,80, 85, 90, or 95 % of its sugars; exhibit a C_3-*endo* pucker in a sufficient amount of its sugars that it can give rise to the RNA-characteristic A-family-type helix; will have no more than 20, 10, 5, 4, 3, 2, or1 sugar which is not a C_3-*endo* pucker structure. These limitations are particularly preferably in the antisense strand;

(5) regardless of the nature of the modification, and even though the RNA agent can contain deoxynucleotides or modified deoxynucleotides, particularly in overhang or other single strand regions, it is preferred that DNA molecules, or any molecule in which more than 50, 60, or 70 % of the nucleotides in the molecule, or more than 50, 60, or 70 % of the nucleotides in a duplexed region are deoxyribonucleotides, or modified deoxyribonucleotides which are deoxy at the 2' position, are excluded from the definition of RNA agent.

A "single strand iRNA agent" as used herein, is an iRNA agent which is made up of a single molecule. It may include a duplexed region, formed by intra-strand pairing, *e.g.*, it may be, or include, a hairpin or pan-handle structure. Single strand iRNA agents are preferably antisense with regard to the target molecule. In preferred embodiments single strand iRNA agents are 5' phosphorylated or include a phosphoryl analog at the 5' prime terminus. 5'-phosphate modifications include those which are compatible with RISC mediated gene silencing. Suitable modifications include: 5'-monophosphate ((HO)2(O)P-O-5'); 5'-diphosphate ((HO)2(O)P-O-P(HO)(O)-O-5'); 5'-triphosphate ((HO)2(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'); 5'-guanosine cap (7-methylated or non-methylated) (7m-G-O-5'-(HO)(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'); 5'-adenosine cap (Appp), and any modified or unmodified nucleotide cap structure (N-O-5'-(HO)(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'); 5'-monothiophosphate (phosphorothioate; (HO)2(S)P-O-5'); 5'-monodithiophosphate (phosphorodithioate; (HO)(HS)(S)P-O-5'), 5'-phosphorothiolate ((HO)2(O)P-S-5'); any additional combination of oxygen/sulfur replaced monophosphate, diphosphate and triphosphates (*e.g.* 5'-alpha-thiotriphosphate, 5'-gamma-thiotriphosphate, etc.), 5'-phosphoramidates ((HO)2(O)P-NH-5', (HO)(NH2)(O)P-O-5', 5'-alkylphosphonates (R=alkyl=methyl, ethyl, isopropyl, propyl, etc., *e.g.* RP(OH)(O)-O-5'-, (OH)2(O)P-5'-CH2-), 5'-alkyletherphosphonates (R=alkylether=methoxymethyl (MeOCH2-), ethoxymethyl, etc., *e.g.* RP(OH)(O)-O-5'-). (These modifications can also be used with the antisense strand of a double stranded iRNA.)

A single strand iRNA agent should be sufficiently long that it can enter the RISC and participate in RISC mediated cleavage of a target mRNA. A single strand iRNA agent is at least 14, and more preferably at least 15, 20, 25, 29, 35, 40, or 50nucleotides in length. It is preferably less than 200, 100, or 60 nucleotides in length.

Hairpin iRNA agents will have a duplex region equal to or at least 17, 18, 19, 29, 21, 22, 23, 24, or 25 nucleotide pairs. The duplex region will preferably be equal to or less than 200, 100, or 50, in length. Preferred ranges for the duplex region are 15-30, 17 to 23, 19 to 23, and 19 to 21 nucleotides pairs in length. The hairpin will preferably have a single strand overhang or terminal unpaired region, preferably the 3', and preferably of the antisense side of the hairpin. Preferred overhangs are 2-3 nucleotides in length.

A "double stranded (ds) iRNA agent" as used herein, is an iRNA agent which includes more than one, and preferably two, strands in which interchain hybridization can form a region of duplex structure.

The antisense strand of a double stranded iRNA agent should be equal to or at least, 14, 15, 16 17, 18, 19, 25, 29, 40, or 60 nucleotides in length. It should be equal to or less than 200, 100, or 50, nucleotides in length. Preferred ranges are 17 to 25, 19 to 23, and 19 to21 nucleotides in length.

The sense strand of a double stranded iRNA agent should be equal to or at least 14, 15, 16 17, 18, 19, 25, 29, 40, or 60 nucleotides in length. It should be equal to or less than 200, 100, or 50, nucleotides in length. Preferred ranges are 17 to 25, 19 to 23, and 19 to21 nucleotides in length.

The double strand portion of a double stranded iRNA agent should be equal to or at least, 14, 15, 16 17, 18, 19, 20, 21, 22, 23, 24, 25, 29, 40, or 60 nucleotide pairs in length. It should be equal to or less than 200, 100, or 50, nucleotides pairs in length. Preferred ranges are 15-30, 17 to 23, 19 to 23, and 19 to 21 nucleotides pairs in length.

In many embodiments, the ds iRNA agent is sufficiently large that it can be cleaved by an endogenous molecule, e.g., by Dicer, to produce smaller ds iRNA agents, e.g., sRNAs agents

It may be desirable to modify one or both of the antisense and sense strands of a double strand iRNA agent. In some cases they will have the same modification or the same class of modification but in other cases the sense and antisense strand will have different modifications,

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e.g., in some cases it is desirable to modify only the sense strand. It may be desirable to modify only the sense strand, *e.g.*, to inactivate it, *e.g.*, the sense strand can be modified in order to inactivate the sense strand and prevent formation of an active sRNA/protein or RISC. This can be accomplished by a modification which prevents 5'-phosphorylation of the sense strand, *e.g.*, by modification with a 5'-O-methyl ribonucleotide (see Nykänen *et al.*, (2001) ATP requirements and small interfering RNA structure in the RNA interference pathway. Cell 107, 309-321.) Other modifications which prevent phosphorylation can also be used, *e.g.*, simply substituting the 5'-OH by H rather than O-Me. Alternatively, a large bulky group may be added to the 5'-phosphate turning it into a phosphodiester linkage, though this may be less desirable as phosphodiesterases can cleave such a linkage and release a functional sRNA 5'-end. Antisense strand modifications include 5' phosphorylation as well as any of the other 5' modifications discussed herein, particularly the 5' modifications discussed above in the section on single stranded iRNA molecules.

It is preferred that the sense and antisense strands be chosen such that the ds iRNA agent includes a single strand or unpaired region at one or both ends of the molecule. Thus, a ds iRNA agent contains sense and antisense strands, preferable paired to contain an overhang, *e.g.*, one or two 5' or 3' overhangs but preferably a 3' overhang of 2-3 nucleotides. Most embodiments will have a 3' overhang. Preferred sRNA agents will have single-stranded overhangs, preferably 3' overhangs, of 1 or preferably 2 or 3 nucleotides in length at each end. The overhangs can be the result of one strand being longer than the other, or the result of two strands of the same length being staggered. 5' ends are preferably phosphorylated.

Preferred lengths for the duplexed region is between 15 and 30, most preferably 18, 19, 20, 21, 22, and 23 nucleotides in length, *e.g.*, in the sRNA agent range discussed above. sRNA agents can resemble in length and structure the natural Dicer processed products from long dsRNAs. Embodiments in which the two strands of the sRNA agent are linked, *e.g.*, covalently linked are also included. Hairpin, or other single strand structures which provide the required double stranded region, and preferably a 3' overhang are also within the invention.

The isolated iRNA agents described herein, including ds iRNA agents and sRNA agents can mediate silencing of a target RNA, *e.g.*, mRNA, *e.g.*, a transcript of a gene that encodes a protein. For convenience, such mRNA is also referred to herein as mRNA to be silenced. Such a gene is also referred to as a target gene. In general, the RNA to be silenced is an endogenous

gene or a pathogen gene. In addition, RNAs other than mRNA, *e.g.*, tRNAs, and viral RNAs, can also be targeted.

As used herein, the phrase "mediates RNAi" refers to the ability to silence, in a sequence specific manner, a target RNA. While not wishing to be bound by theory, it is believed that silencing uses the RNAi machinery or process and a guide RNA, *e.g.*, an sRNA agent of 21 to 23 nucleotides.

As used herein, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity such that stable and specific binding occurs between a compound of the invention and a target RNA molecule. Specific binding requires a sufficient degree of complementarity to avoid non-specific binding of the oligomeric compound to non-target sequences under conditions in which specific binding is desired, *i.e.*, under physiological conditions in the case of *in vivo* assays or therapeutic treatment, or in the case of *in vitro* assays, under conditions in which the assays are performed. The non-target sequences typically differ by at least 5 nucleotides.

In one embodiment, an iRNA agent is "sufficiently complementary" to a target RNA, *e.g.*, a target mRNA, such that the iRNA agent silences production of protein encoded by the target mRNA. In another embodiment, the iRNA agent is "exactly complementary" (excluding the RRMS containing subunit(s))to a target RNA, *e.g.*, the target RNA and the iRNA agent anneal, preferably to form a hybrid made exclusively of Watson-Crick basepairs in the region of exact complementarity. A "sufficiently complementary" target RNA can include an internal region (*e.g.*, of at least 10 nucleotides) that is exactly complementary to a target RNA. Moreover, in some embodiments, the iRNA agent specifically discriminates a single-nucleotide difference. In this case, the iRNA agent only mediates RNAi if exact complementary is found in the region (*e.g.*, within 7 nucleotides of) the single-nucleotide difference.

As used herein, the term "oligonucleotide" refers to a nucleic acid molecule (RNA or DNA) preferably of length less than 100, 200, 300, or 400 nucleotides.

RNA agents discussed herein include otherwise unmodified RNA as well as RNA which have been modified, *e.g.*, to improve efficacy, and polymers of nucleoside surrogates. Unmodified RNA refers to a molecule in which the components of the nucleic acid, namely sugars, bases, and phosphate moieties, are the same or essentially the same as that which occur in nature, preferably as occur naturally in the human body. The art has referred to rare or unusual,

but naturally occurring, RNAs as modified RNAs, see, *e.g.*, Limbach *et al.*, (1994) Summary: the modified nucleosides of RNA, Nucleic Acids Res. 22: 2183-2196. Such rare or unusual RNAs, often termed modified RNAs (apparently because the are typically the result of a post transcriptionally modification) are within the term unmodified RNA, as used herein. Modified

5   RNA as used herein refers to a molecule in which one or more of the components of the nucleic acid, namely sugars, bases, and phosphate moieties, are different from that which occur in nature, preferably different from that which occurs in the human body. While they are referred to as modified "RNAs," they will of course, because of the modification, include molecules which are not RNAs. Nucleoside surrogates are molecules in which the ribophosphate backbone

10 is replaced with a non-ribophosphate construct that allows the bases to the presented in the correct spatial relationship such that hybridization is substantially similar to what is seen with a ribophosphate backbone, *e.g.*, non-charged mimics of the ribophosphate backbone. Examples of all of the above are discussed herein.

Much of the discussion below refers to single strand molecules. In many embodiments of
15 the invention a double stranded iRNA agent, *e.g.*, a partially double stranded iRNA agent, is required or preferred. Thus, it is understood that that double stranded structures (*e.g.* where two separate molecules are contacted to form the double stranded region or where the double stranded region is formed by intramolecular pairing (*e.g.*, a hairpin structure)) made of the single stranded structures described below are within the invention. Preferred lengths are described
20 elsewhere herein.

As nucleic acids are polymers of subunits or monomers, many of the modifications described below occur at a position which is repeated within a nucleic acid, *e.g.*, a modification of a base, or a phosphate moiety, or the non-linking O of a phosphate moiety. In some cases the modification will occur at all of the subject positions in the nucleic acid but in many, and
25 infact in most cases it will not. By way of example, a modification may only occur at a 3' or 5' terminal position, may only occur in a terminal regions, *e.g.* at a position on a terminal nucleotide or in the last 2, 3, 4, 5, or 10 nucleotides of a strand. A modification may occur in a double strand region, a single strand region, or in both. A modification may occur only in the double strand region of an RNA or may only occur in a single strand region of an RNA. *E.g.*, a
30 phosphorothioate modification at a non-linking O position may only occur at one or both termini, may only occur in a terminal regions, *e.g.*, at a position on a terminal nucleotide or in the last 2, 3, 4, 5, or 10 nucleotides of a strand, or may occur in double strand and single strand regions, particularly at termini. The 5' end or ends can be phosphorylated.

In some embodiments it is particularly preferred, *e.g.*, to enhance stability, to include particular bases in overhangs, or to include modified nucleotides or nucleotide surrogates, in single strand overhangs, *e.g.*, in a 5' or 3' overhang, or in both. *E.g.*, it can be desirable to include purine nucleotides in overhangs. In some embodiments all or some of the bases in a 3' or 5' overhang will be modified, *e.g.*, with a modification described herein. Modifications can include, *e.g.*, the use of modifications at the 2' OH group of the ribose sugar, *e.g.*, the use of deoxyribonucleotides, *e.g.*, deoxythymidine, instead of ribonucleotides, and modifications in the phosphate group, *e.g.*, phosphothioate modifications. Overhangs need not be homologous with the target sequence.

Modifications and nucleotide surrogates are discussed below.
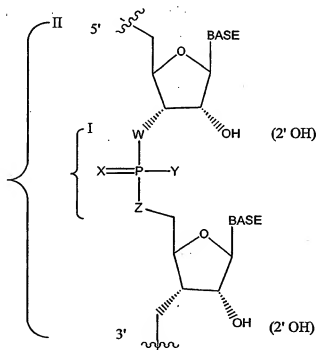
FORMULA 1

The scaffold presented above in Formula 1 represents a portion of a ribonucleic acid. The basic components are the ribose sugar, the base, the terminal phosphates, and phosphate internucleotide linkers. Where the bases are naturally occurring bases, *e.g.*, adenine, uracil,

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guanine or cytosine, the sugars are the unmodified 2′ hydroxyl ribose sugar (as depicted) and W, X, Y, and Z are all O, Formula 1 represents a naturally occurring unmodified oligoribonucleotide.

Unmodified oligoribonucleotides may be less than optimal in some applications, e.g., unmodified oligoribonucleotides can be prone to degradation by e.g., cellular nucleases. Nucleases can hydrolyze nucleic acid phosphodiester bonds. However, chemical modifications to one or more of the above RNA components can confer improved properties, and, e.g., can render oligoribonucleotides more stable to nucleases. Umodified oligoribonucleotides may also be less than optimal in terms of offering tethering points for attaching ligands or other moieties to an iRNA agent.

Modified nucleic acids and nucleotide surrogates can include one or more of:

(i) alteration, e.g., replacement, of one or both of the non-linking (X and Y) phosphate oxygens and/or of one or more of the linking (W and Z) phosphate oxygens (When the phosphate is in the terminal position, one of the positions W or Z will not link the phosphate to an additional element in a naturally occurring ribonucleic acid. However, for simplicity of terminology, except where otherwise noted, the W position at the 5′ end of a nucleic acid and the terminal Z position at the 3′ end of a nucleic acid, are within the term "linking phosphate oxygens" as used herein.);

(ii) alteration, e.g., replacement, of a constituent of the ribose sugar, e.g., of the 2′ hydroxyl on the ribose sugar, or wholesale replacement of the ribose sugar with a structure other than ribose, e.g., as described herein;

(iii) wholesale replacement of the phosphate moiety (bracket I) with "dephospho" linkers;

(iv) modification or replacement of a naturally occurring base;

(v) replacement or modification of the ribose-phosphate backbone (bracket II);

(vi) modification of the 3' end or 5' end of the RNA, e.g., removal, modification or replacement of a terminal phosphate group or conjugation of a moiety, e.g. a fluorescently labeled moiety, to either the 3′ or 5′ end of RNA.

The terms replacement, modification, alteration, and the like, as used in this context, do not imply any process limitation, e.g., modification does not mean that one must start with a

reference or naturally occurring ribonucleic acid and modify it to produce a modified ribonucleic acid bur rather modified simply indicates a difference from a naturally occurring molecule.

It is understood that the actual electronic structure of some chemical entities cannot be adequately represented by only one canonical form (*i.e.* Lewis structure). While not wishing to be bound by theory, the actual structure can instead be some hybrid or weighted average of two or more canonical forms, known collectively as resonance forms or structures. Resonance structures are not discrete chemical entities and exist only on paper. They differ from one another only in the placement or "localization" of the bonding and nonbonding electrons for a particular chemical entity. It can be possible for one resonance structure to contribute to a greater extent to the hybrid than the others. Thus, the written and graphical descriptions of the embodiments of the present invention are made in terms of what the art recognizes as the predominant resonance form for a particular species. For example, any phosphoroamidate (replacement of a nonlinking oxygen with nitrogen) would be represented by X = O and Y = N in the above figure.

Specific modifications are discussed in more detail below.

The Phosphate Group

The phosphate group is a negatively charged species. The charge is distributed equally over the two non-linking oxygen atoms (*i.e.*, X and Y in Formula 1 above). However, the phosphate group can be modified by replacing one of the oxygens with a different substituent. One result of this modification to RNA phosphate backbones can be increased resistance of the oligoribonucleotide to nucleolytic breakdown. Thus while not wishing to be bound by theory, it can be desirable in some embodiments to introduce alterations which result in either an uncharged linker or a charged linker with unsymmetrical charge distribution.

Examples of modified phosphate groups include phosphorothioate, phosphoroselenates, borano phosphates, borano phosphate esters, hydrogen phosphonates, phosphoroamidates, alkyl or aryl phosphonates and phosphotriesters. Phosphorodithioates have both non-linking oxygens replaced by sulfur. Unlike the situation where only one of X or Y is altered, the phosphorus center in the phosphorodithioates is achiral which precludes the formation of oligoribonucleotides diastereomers. Diastereomer formation can result in a preparation in which the individual diastereomers exhibit varying resistance to nucleases. Further, the hybridization affinity of RNA containing chiral phosphate groups can be lower relative to the corresponding

unmodified RNA species. Thus, while not wishing to be bound by theory, modifications to both X and Y which eliminate the chiral center, *e.g.* phosphorodithioate formation, may be desirable in that they cannot produce diastereomer mixtures. Thus, X can be any one of S, Se, B, C, H, N, or OR (R is alkyl or aryl). Thus Y can be any one of S, Se, B, C, H, N, or OR (R is alkyl or aryl). Replacement of X and/or Y with sulfur is preferred.

The phosphate linker can also be modified by replacement of a linking oxygen (*i.e.*, W or Z in Formula 1) with nitrogen (bridged phosphoroamidates), sulfur (bridged phosphorothioates) and carbon (bridged methylenephosphonates). The replacement can occur at a terminal oxygen (position W (3′) or position Z (5′). Replacement of W with carbon or Z with nitrogen is preferred.

Candidate agents can be evaluated for suitability as described below.

The Sugar Group

A modified RNA can include modification of all or some of the sugar groups of the ribonucleic acid. *E.g.*, the 2′ hydroxyl group (OH) can be modified or replaced with a number of different "oxy" or "deoxy" substituents. While not being bound by theory, enhanced stability is expected since the hydroxyl can no longer be deprotonated to form a 2′ alkoxide ion. The 2′ alkoxide can catalyze degradation by intramolecular nucleophilic attack on the linker phosphorus atom. Again, while not wishing to be bound by theory, it can be desirable to some embodiments to introduce alterations in which alkoxide formation at the 2′ position is not possible.

Examples of "oxy"-2′ hydroxyl group modifications include alkoxy or aryloxy (OR, *e.g.*, R = H, alkyl, cycloalkyl, aryl, aralkyl, heteroaryl or sugar); polyethyleneglycols (PEG), $O(CH_2CH_2O)_nCH_2CH_2OR$; "locked" nucleic acids (LNA) in which the 2′ hydroxyl is connected, *e.g.*, by a methylene bridge, to the 4′ carbon of the same ribose sugar; O-AMINE (AMINE = NH_2; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamino) and aminoalkoxy, $O(CH_2)_nAMINE$, (*e.g.*, AMINE = NH_2; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamino). It is noteworthy that oligonucleotides containing only the methoxyethyl group (MOE), ($OCH_2CH_2OCH_3$, a PEG derivative), exhibit nuclease stabilities comparable to those modified with the robust phosphorothioate modification.

"Deoxy" modifications include hydrogen (*i.e.* deoxyribose sugars, which are of particular relevance to the overhang portions of partially ds RNA); halo (*e.g.*, fluoro); amino (*e.g.* NH$_2$; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, or amino acid); NH(CH$_2$CH$_2$NH)$_n$CH$_2$CH$_2$-AMINE (AMINE = NH$_2$; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino,or diheteroaryl amino), -NHC(O)R (R = alkyl, cycloalkyl, aryl, aralkyl, heteroaryl or sugar), cyano; mercapto; alkyl-thio-alkyl; thioalkoxy; and alkyl, cycloalkyl, aryl, alkenyl and alkynyl, which may be optionally substituted with *e.g.*, an amino functionality. Preferred substitutents are 2'-methoxyethyl, 2'-OCH3, 2'-O-allyl, 2'-C- allyl, and 2'-fluoro.

The sugar group can also contain one or more carbons that possess the opposite stereochemical configuration than that of the corresponding carbon in ribose. Thus, a modified RNA can include nucleotides containing *e.g.*, arabinose, as the sugar.

Modified RNAs can also include "abasic" sugars, which lack a nucleobase at C-1'. These abasic sugars can also be further contain modifications at one or more of the constituent sugar atoms.

To maximize nuclease resistance, the 2' modifications can be used in combination with one or more phosphate linker modifications (*e.g.*, phosphorothioate). The so-called "chimeric" oligonucleotides are those that contain two or more different modifications.

The modificaton can also entail the wholesale replacement of a ribose structure with another entity at one or more sites in the iRNA agent. These modifications are described in section entitled Ribose Replacements for RRMSs.

Candidate modifications can be evaluated as described below.

Replacement of the Phosphate Group

The phosphate group can be replaced by non-phosphorus containing connectors (*cf.* Bracket I in Formula 1 above). While not wishing to be bound by theory, it is believed that since the charged phosphodiester group is the reaction center in nucleolytic degradation, its replacement with neutral structural mimics should impart enhanced nuclease stability. Again, while not wishing to be bound by theory, it can be desirable, in some embodiment, to introduce alterations in which the charged phosphate group is replaced by a neutral moiety.

Examples of moieties which can replace the phosphate group include siloxane, carbonate, carboxymethyl, carbamate, amide, thioether, ethylene oxide linker, sulfonate, sulfonamide, thioformacetal, formacetal, oxime, methyleneimino, methylenemethylimino, methylenehydrazo, methylenedimethylhydrazo and methyleneoxymethylimino. Preferred replacements include the methylenecarbonylamino and methylenemethylimino groups.

Candidate modifications can be evaluated as described below.

Replacement of Ribophosphate Backbone

Oligonucleotide- mimicking scaffolds can also be constructed wherein the phosphate linker and ribose sugar are replaced by nuclease resistant nucleoside or nucleotide surrogates (see Bracket II of Formula 1 above). While not wishing to be bound by theory, it is believed that the absence of a repetitively charged backbone diminishes binding to proteins that recognize polyanions (e.g. nucleases). Again, while not wishing to be bound by theory, it can be desirable in some embodiment, to introduce alterations in which the bases are tethered by a neutral surrogate backbone.

Examples include the mophilino, cyclobutyl, pyrrolidine and peptide nucleic acid (PNA) nucleoside surrogates. A preferred surrogate is a PNA surrogate.

Candidate modifications can be evaluated as described below.

Terminal Modifications

The 3′ and 5′ ends of an oligonucleotide can be modified. Such modifications can be at the 3′ end, 5′ end or both ends of the molecule. They can include modification or replacement of an entire terminal phosphate or of one or more of the atoms of the phosphate group. E.g., the 3′ and 5′ ends of an oligonucleotide can be conjugated to other functional molecular entities such as labeling moieties, e.g., fluorophores (e.g., pyrene, TAMRA, fluorescein, Cy3 or Cy5 dyes) or protecting groups (based e.g., on sulfur, silicon, boron or ester). The functional molecular entities can be attached to the sugar through a phosphate group and/or a spacer. The terminal atom of the spacer can connect to or replace the linking atom of the phosphate group or the C-3′ or C-5′ O, N, S or C group of the sugar. Alternatively, the spacer can connect to or replace the terminal atom of a nucleotide surrogate (e.g., PNAs). These spacers or linkers can include e.g., -(CH$_2$)$_n$-, -(CH$_2$)$_n$N-, -(CH$_2$)$_n$O-, -(CH$_2$)$_n$S-, O(CH$_2$CH$_2$O)$_n$CH$_2$CH$_2$OH (e.g., n = 3 or 6), abasic sugars, amide, carboxy, amine, oxyamine, oximine, thioether, disulfide, thiourea, sulfonamide,

or morpholino, or biotin and fluorescein reagents. When a spacer/phosphate-functional molecular entity-spacer/phosphate array is interposed between two strands of iRNA agents, this array can substitute for a hairpin RNA loop in a hairpin-type RNA agent. The 3' end can be an – OH group. While not wishing to be bound by theory, it is believed that conjugation of certain moieties can improve transport, hybridization, and specificity properties. Again, while not wishing to be bound by theory, it may be desirable to introduce terminal alterations that improve nuclease resistance. Other examples of terminal modifications include dyes, intercalating agents (e.g. acridines), cross-linkers (e.g. psoralene, mitomycin C), porphyrins (TPPC4, texaphyrin, Sapphyrin), polycyclic aromatic hydrocarbons (e.g., phenazine, dihydrophenazine), artificial endonucleases (e.g. EDTA), lipophilic carriers (e.g., cholesterol, cholic acid, adamantane acetic acid, 1-pyrene butyric acid, dihydrotestosterone, 1,3-Bis-O(hexadecyl)glycerol, geranyloxyhexyl group, hexadecylglycerol, borneol, menthol, 1,3-propanediol, heptadecyl group, palmitic acid, myristic acid,O3-(oleoyl)lithocholic acid, O3-(oleoyl)cholenic acid, dimethoxytrityl, or phenoxazine)and peptide conjugates (e.g., antennapedia peptide, Tat peptide), alkylating agents, phosphate, amino, mercapto, PEG (e.g., PEG-40K), MPEG, [MPEG]$_2$, polyamino, alkyl, substituted alkyl, radiolabeled markers, enzymes, haptens (e.g. biotin), transport/absorption facilitators (e.g., aspirin, vitamin E, folic acid), synthetic ribonucleases (e.g., imidazole, bisimidazole, histamine, imidazole clusters, acridine-imidazole conjugates, Eu3+ complexes of tetraazamacrocycles).

Terminal modifications can be added for a number of reasons, including as discussed elsewhere herein to modulate activity or to modulate resistance to degradation. Terminal modifications useful for modulating activity include modification of the 5' end with phosphate or phosphate analogs. E.g., in preferred embodiments iRNA agents, especially antisense strands, are 5' phosphorylated or include a phosphoryl analog at the 5' prime terminus. 5'-phosphate modifications include those which are compatible with RISC mediated gene silencing. Suitable modifications include: 5'-monophosphate ((HO)2(O)P-O-5'); 5'-diphosphate ((HO)2(O)P-O-P(HO)(O)-O-5'); 5'-triphosphate ((HO)2(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'); 5'-guanosine cap (7-methylated or non-methylated) (7m-G-O-5'-(HO)(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'); 5'-adenosine cap (Appp), and any modified or unmodified nucleotide cap structure (N-O-5'-(HO)(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'); 5'-monothiophosphate (phosphorothioate; (HO)2(S)P-O-5'); 5'-monodithiophosphate (phosphorodithioate; (HO)(HS)(S)P-O-5'), 5'-phosphorothiolate ((HO)2(O)P-S-5'); any additional combination of oxgen/sulfur replaced monophosphate, diphosphate and triphosphates (e.g. 5'-alpha-thiotriphosphate, 5'-gamma-

thiotriphosphate, etc.), 5'-phosphoramidates ((HO)2(O)P-NH-5', (HO)(NH2)(O)P-O-5'), 5'-alkylphosphonates (R=alkyl=methyl, ethyl, isopropyl, propyl, etc., *e.g.* RP(OH)(O)-O-5'-, (OH)2(O)P-5'-CH2-), 5'-alkyletherphosphonates (R=alkylether=methoxymethyl (MeOCH2-), ethoxymethyl, etc., *e.g.* RP(OH)(O)-O-5'-).

5   Terminal modifications can also be useful for monitoring distribution, and in such cases the preferred groups to be added include fluorophores, *e.g.*, fluorscein or an Alexa dye, *e.g.*, Alexa 488. Terminal modifications can also be useful for enhancing uptake, useful modifications for this include cholesterol. Terminal modifications can also be useful for cross-linking an RNA agent to another moiety; modifications useful for this include mitomycin C.

10   Candidate modifications can be evaluated as described below.

The Bases

         Adenine, guanine, cytosine and uracil are the most common bases found in RNA. These bases can be modified or replaced to provide RNA's having improved properties. *E.g.*, nuclease resistant oligoribonucleotides can be prepared with these bases or with synthetic and natural
15 nucleobases (*e.g.*, inosine, thymine, xanthine, hypoxanthine, nubularine, isoguanisine, or tubercidine) and any one of the above modifications. Alternatively, substituted or modified analogs of any of the above bases, e.g., "unusual bases" and "universal bases," can be employed. Examples include without limitation 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 5-halouracil
20 and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 5-halouracil, 5-(2-aminopropyl)uracil, 5-amino allyl uracil, 8-halo, amino, thiol, thioalkyl, hydroxyl and other 8-substituted adenines and guanines, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine, 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-
25 aminopropyladenine, 5-propynyluracil and 5-propynylcytosine, dihydrouracil, 3-deaza-5-azacytosine, 2-aminopurine, 5-alkyluracil, 7-alkylguanine, 5-alkyl cytosine,7-deazaadenine, N6, N6-dimethyladenine, 2,6-diaminopurine, 5-amino-allyl-uracil, N3-methyluracil, substituted 1,2,4-triazoles, 2-pyridinone, 5-nitroindole, 3-nitropyrrole, 5-methoxyuracil, uracil-5-oxyacetic acid, 5-methoxycarbonylmethyluracil, 5-methyl-2-thiouracil, 5-methoxycarbonylmethyl-2-
30 thiouracil, 5-methylaminomethyl-2-thiouracil, 3-(3-amino-3carboxypropyl)uracil, 3-methylcytosine, 5-methylcytosine, N^4-acetyl cytosine, 2-thiocytosine, N6-methyladenine, N6-isopentyladenine, 2-methylthio-N6-isopentenyladenine, N-methylguanines, or O-alkylated bases.

Further purines and pyrimidines include those disclosed in U.S. Pat. No. 3,687,808, those disclosed in the Concise Encyclopedia Of Polymer Science And Engineering, pages 858-859, Kroschwitz, J. I., ed. John Wiley & Sons, 1990, and those disclosed by Englisch *et al.*, Angewandte Chemie, International Edition, 1991, 30, 613.

Generally, base changes are less preferred for promoting stability, but they can be useful for other reasons, *e.g.*, some, *e.g.*, 2,6-diaminopurine and 2 amino purine, are fluorescent. Modified bases can reduce target specificity. This should be taken into consideration in the design of iRNA agents.

Candidate modifications can be evaluated as described below.

Evaluation of Candidate RNA's

One can evaluate a candidate RNA agent, *e.g.*, a modified RNA, for a selected property by exposing the agent or modified molecule and a control molecule to the appropriate conditions and evaluating for the presence of the selected property. For example, resistance to a degradent can be evaluated as follows. A candidate modified RNA (and preferably a control molecule, usually the unmodified form) can be exposed to degradative conditions, *e.g.*, exposed to a milieu, which includes a degradative agent, *e.g.*, a nuclease. *E.g.*, one can use a biological sample, *e.g.*, one that is similar to a milieu, which might be encountered, in therapeutic use, *e.g.*, blood or a cellular fraction, *e.g.*, a cell-free homogenate or disrupted cells. The candidate and control could then be evaluated for resistance to degradation by any of a number of approaches. For example, the candidate and control could be labeled, preferably prior to exposure, with, *e.g.*, a radioactive or enzymatic label, or a fluorescent label, such as Cy3 or Cy5. Control and modified RNA's can be incubated with the degradative agent, and optionally a control, *e.g.*, an inactivated, *e.g.*, heat inactivated, degradative agent. A physical parameter, *e.g.*, size, of the modified and control molecules are then determined. They can be determined by a physical method, *e.g.*, by polyacrylamide gel electrophoresis or a sizing column, to assess whether the molecule has maintained its original length, or assessed functionally. Alternatively, Northern blot analysis can be used to assay the length of an unlabeled modified molecule.

A functional assay can also be used to evaluate the candidate agent. A functional assay can be applied initially or after an earlier non-functional assay, (*e.g.*, assay for resistance to degradation) to determine if the modification alters the ability of the molecule to silence gene expression. For example, a cell, *e.g.*, a mammalian cell, such as a mouse or human cell, can be

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co-transfected with a plasmid expressing a fluorescent protein, e.g., GFP, and a candidate RNA agent homologous to the transcript encoding the fluorescent protein (see, e.g., WO 00/44914). For example, a modified dsRNA homologous to the GFP mRNA can be assayed for the ability to inhibit GFP expression by monitoring for a decrease in cell fluorescence, as compared to a control cell, in which the transfection did not include the candidate dsRNA, e.g., controls with no agent added and/or controls with a non-modified RNA added. Efficacy of the candidate agent on gene expression can be assessed by comparing cell fluorescence in the presence of the modified and unmodified dsRNA agents.

In an alternative functional assay, a candidate dsRNA agent homologous to an endogenous mouse gene, preferably a maternally expressed gene, such as c-mos, can be injected into an immature mouse oocyte to assess the ability of the agent to inhibit gene expression in vivo (see, e.g., WO 01/36646). A phenotype of the oocyte, e.g., the ability to maintain arrest in metaphase II, can be monitored as an indicator that the agent is inhibiting expression. For example, cleavage of c-mos mRNA by a dsRNA agent would cause the oocyte to exit metaphase arrest and initiate parthenogenetic development (Colledge et al. Nature 370: 65-68, 1994; Hashimoto et al. Nature, 370:68-71, 1994). The effect of the modified agent on target RNA levels can be verified by Northern blot to assay for a decrease in the level of target mRNA, or by Western blot to assay for a decrease in the level of target protein, as compared to a negative control. Controls can include cells in which with no agent is added and/or cells in which a non-modified RNA is added.

References

General References

The oligoribonucleotides and oligoribonucleosides used in accordance with this invention may be with solid phase synthesis, see for example "Oligonucleotide synthesis, a practical approach", Ed. M. J. Gait, IRL Press, 1984; "Oligonucleotides and Analogues, A Practical Approach", Ed. F. Eckstein, IRL Press, 1991 (especially Chapter 1, Modern machine-aided methods of oligodeoxyribonucleotide synthesis, Chapter 2, Oligoribonucleotide synthesis, Chapter 3, 2'-O--Methyloligoribonucleotide- s: synthesis and applications, Chapter 4, Phosphorothioate oligonucleotides, Chapter 5, Synthesis of oligonucleotide phosphorodithioates, Chapter 6, Synthesis of oligo-2'-deoxyribonucleoside methylphosphonates, and. Chapter 7, Oligodeoxynucleotides containing modified bases. Other particularly useful synthetic procedures, reagents, blocking groups and reaction conditions are described in Martin, P., *Helv.*

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Chim. Acta, **1995**, *78*, 486-504; Beaucage, S. L. and Iyer, R. P., *Tetrahedron*, **1992**, *48*, 2223-2311 and Beaucage, S. L. and Iyer, R. P., *Tetrahedron*, **1993**, *49*, 6123-6194, or references referred to therein.

Modification described in WO 00/44895, WO01/75164, or WO02/44321 can be used herein.

The disclosure of all publications, patents, and published patent applications listed herein are hereby incorporated by reference.

Phosphate Group References

The preparation of phosphinate oligoribonucleotides is described in U.S. Pat. No. 5,508,270. The preparation of alkyl phosphonate oligoribonucleotides is described in U.S. Pat. No. 4,469,863. The preparation of phosphoramidite oligoribonucleotides is described in U.S. Pat. No. 5,256,775 or U.S. Pat. No. 5,366,878. The preparation of phosphotriester oligoribonucleotides is described in U.S. Pat. No. 5,023,243. The preparation of borano phosphate oligoribonucleotide is described in U.S. Pat. Nos. 5,130,302 and 5,177,198. The preparation of 3'-Deoxy-3'-amino phosphoramidate oligoribonucleotides is described in U.S. Pat. No. 5,476,925. 3'-Deoxy-3'-methylenephosphonate oligoribonucleotides is described in An, H, *et al. J. Org. Chem.* **2001**, *66*, 2789-2801. Preparation of sulfur bridged nucleotides is described in Sproat *et al. Nucleosides Nucleotides* **1988**, *7*,651 and Crosstick *et al. Tetrahedron Lett.* **1989**, *30*, 4693.

Sugar Group References

Modifications to the 2' modifications can be found in Verma, S. *et al. Annu. Rev. Biochem.* **1998**, *67*, 99-134 and all references therein. Specific modifications to the ribose can be found in the following references: 2'-fluoro (Kawasaki et. al., *J. Med. Chem.*, **1993**, *36*, 831-841), 2'-MOE (Martin, P. *Helv. Chim. Acta* **1996**, *79*, 1930-1938), "LNA" (Wengel, J. *Acc. Chem. Res.* **1999**, *32*, 301-310).

Replacement of the Phosphate Group References

Methylenemethylimino linked oligoribonucleosides, also identified herein as MMI linked oligoribonucleosides, methylenedimethylhydrazo linked oligoribonucleosides, also identified herein as MDH linked oligoribonucleosides, and methylenecarbonylamino linked

oligonucleosides, also identified herein as amide-3 linked oligoribonucleosides, and methyleneaminocarbonyl linked oligonucleosides, also identified herein as amide-4 linked oligoribonucleosides as well as mixed backbone compounds having, as for instance, alternating MMI and PO or PS linkages can be prepared as is described in U.S. Pat. Nos. 5,378,825, 5,386,023, 5,489,677 and in published PCT applications PCT/US92/04294 and PCT/US92/04305 (published as WO 92/20822 WO and 92/20823, respectively). Formacetal and thioformacetal linked oligoribonucleosides can be prepared as is described in U.S. Pat. Nos. 5,264,562 and 5,264,564. Ethylene oxide linked oligoribonucleosides can be prepared as is described in U.S. Pat. No. 5,223,618. Siloxane replacements are described in Cormier, J.F. *et al.* *Nucleic Acids Res.* 1988, *16*, 4583. Carbonate replacements are described in Tittensor, J.R. *J. Chem. Soc. C* 1971, 1933. Carboxymethyl replacements are described in Edge, M.D. *et al. J. Chem. Soc. Perkin Trans. 1* 1972, 1991. Carbamate replacements are described in Stirchak, E.P. Nucleic Acids Res. 1989, 17, 6129.

Replacement of the Phosphate-Ribose Backbone References

Cyclobutyl sugar surrogate compounds can be prepared as is described in U.S. Pat. No. 5,359,044. Pyrrolidine sugar surrogate can be prepared as is described in U.S. Pat. No. 5,519,134. Morpholino sugar surrogates can be prepared as is described in U.S. Pat. Nos. 5,142,047 and 5,235,033, and other related patent disclosures. Peptide Nucleic Acids (PNAs) are known per se and can be prepared in accordance with any of the various procedures referred to in Peptide Nucleic Acids (PNA): Synthesis, Properties and Potential Applications, Bioorganic & Medicinal Chemistry, 1996, 4, 5-23. They may also be prepared in accordance with U.S. Pat. No. 5,539,083.

Terminal Modification References

Terminal modifications are described in Manoharan, M. *et al. Antisense and Nucleic Acid Drug Development 12*, 103-128 (2002) and references therein.

Bases References

N-2 substituted purine nucleoside amidites can be prepared as is described in U.S. Pat. No. 5,459,255. 3-Deaza purine nucleoside amidites can be prepared as is described in U.S. Pat. No. 5,457,191. 5,6-Substituted pyrimidine nucleoside amidites can be prepared as is described in U.S. Pat. No. 5,614,617. 5-Propynyl pyrimidine nucleoside amidites can be prepared as is

described in U.S. Pat. No. 5,484,908. Additional references can be disclosed in the above section on base modifications.

Preferred iRNA Agents

Preferred RNA agents have the following structure (see Formula 2 below):
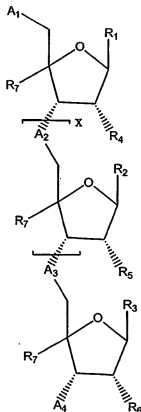
FORMULA 2

Referring to Formula 2 above, R^1, R^2, and R^3 are each, independently, H, (i.e. abasic nucleotides), adenine, guanine, cytosine and uracil, inosine, thymine, xanthine, hypoxanthine, nubularine, tubercidine, isoguanisine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 5-halouracil, 5-(2-aminopropyl)uracil, 5-amino allyl uracil, 8-halo,

43

amino, thiol, thioalkyl, hydroxyl and other 8-substituted adenines and guanines, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine, 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine, dihydrouracil, 3-deaza-5-azacytosine, 2-aminopurine, 5-alkyluracil, 7-alkylguanine, 5-alkyl cytosine,7-deazaadenine, 7-deazaguanine, N6, N6-dimethyladenine, 2,6-diaminopurine, 5-amino-allyl-uracil, N3-methyluracil, substituted 1,2,4-triazoles, 2-pyridinone, 5-nitroindole, 3-nitropyrrole, 5-methoxyuracil, uracil-5-oxyacetic acid, 5-methoxycarbonylmethyluracil, 5-methyl-2-thiouracil, 5-methoxycarbonylmethyl-2-thiouracil, 5-methylaminomethyl-2-thiouracil, 3-(3-amino-3carboxypropyl)uracil, 3-methylcytosine, 5-methylcytosine, N^4-acetyl cytosine, 2-thiocytosine, N6-methyladenine, N6-isopentyladenine, 2-methylthio-N6-isopentenyladenine, N-methylguanines, or O-alkylated bases.

R^4, R^5, and R^6 are each, independently, OR^8, $O(CH_2CH_2O)_mCH_2CH_2OR^8$; $O(CH_2)_nR^9$; $O(CH_2)_nOR^9$, H; halo; NH_2; NHR^8; $N(R^8)_2$; $NH(CH_2CH_2NH)_mCH_2CH_2NHR^9$; $NHC(O)R^8$; ; cyano; mercapto, SR^8; alkyl-thio-alkyl; alkyl, aralkyl, cycloalkyl, aryl, heteroaryl, alkenyl, alkynyl, each of which may be optionally substituted with halo, hydroxy, oxo, nitro, haloalkyl, alkyl, alkaryl, aryl, aralkyl, alkoxy, aryloxy, amino, alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, acylamino, alkylcarbamoyl, arylcarbamoyl, aminoalkyl, alkoxycarbonyl, carboxy, hydroxyalkyl, alkanesulfonyl, alkanesulfonamido, arenesulfonamido, aralkylsulfonamido, alkylcarbonyl, acyloxy, cyano, or ureido; or R^4, R^5, or R^6 together combine with R^7 to form an [-O-CH_2-] covalently bound bridge between the sugar 2' and 4' carbons.

A^1 is:
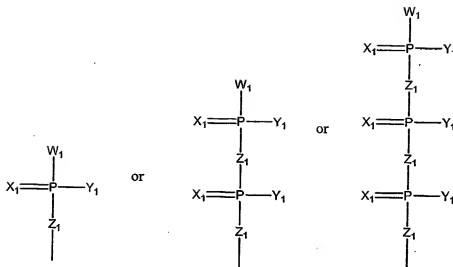
; H; OH; OCH_3; W^1; an abasic nucleotide; or absent;

(a preferred A1 , especially with regard to anti-sense strands, is chosen from 5'-monophosphate $((HO)_2(O)P\text{-}O\text{-}5')$, 5'-diphosphate $((HO)_2(O)P\text{-}O\text{-}P(HO)(O)\text{-}O\text{-}5')$, 5'-triphosphate $((HO)_2(O)P\text{-}O\text{-}(HO)(O)P\text{-}O\text{-}P(HO)(O)\text{-}O\text{-}5')$, 5'-guanosine cap (7-methylated or non-methylated) $(7m\text{-}G\text{-}O\text{-}5'\text{-}(HO)(O)P\text{-}O\text{-}(HO)(O)P\text{-}O\text{-}P(HO)(O)\text{-}O\text{-}5')$, 5'-adenosine cap (Appp), and any modified or unmodified nucleotide cap structure $(N\text{-}O\text{-}5'\text{-}(HO)(O)P\text{-}O\text{-}(HO)(O)P\text{-}O\text{-}P(HO)(O)\text{-}O\text{-}5')$, 5'-monothiophosphate (phosphorothioate; $(HO)_2(S)P\text{-}O\text{-}5')$, 5'-monodithiophosphate (phosphorodithioate; $(HO)(HS)(S)P\text{-}O\text{-}5')$, 5'-phosphorothiolate $((HO)_2(O)P\text{-}S\text{-}5')$; any additional combination of oxgen/sulfur replaced monophosphate, diphosphate and triphosphates (*e.g.* 5'-alpha-thiotriphosphate, 5'-gamma-thiotriphosphate, etc.), 5'-phosphoramidates $((HO)_2(O)P\text{-}NH\text{-}5'$, $(HO)(NH_2)(O)P\text{-}O\text{-}5')$, 5'-alkylphosphonates (R=alkyl=methyl, ethyl, isopropyl, propyl, etc., *e.g.* $RP(OH)(O)\text{-}O\text{-}5'\text{-}$, $(OH_2)(O)P\text{-}5'\text{-}CH_2\text{-})$, 5'-alkyletherphosphonates (R=alkylether=methoxymethyl $(MeOCH_2\text{-})$, ethoxymethyl, etc., *e.g.* $RP(OH)(O)\text{-}O\text{-}5'\text{-}))$.

A^2 is:

$$X_2 = \overset{\overset{\displaystyle |}{Z_2}}{\underset{\underset{\displaystyle |}{Z_2}}{P}} - Y_2$$

A^3 is:

$$X_3 = \overset{\overset{\displaystyle |}{Z_3}}{\underset{\underset{\displaystyle |}{Z_3}}{P}} - Y_3$$

5   ; and

A^4 is:

; H; Z^4; an inverted nucleotide; an abasic nucleotide; or absent.

W^1 is OH, $(CH_2)_nR^{10}$, $(CH_2)_nNHR^{10}$, $(CH_2)_n OR^{10}$, $(CH_2)_n SR^{10}$; $O(CH_2)_nR^{10}$;

$O(CH_2)_nOR^{10}$, $O(CH_2)_nNR^{10}$, $O(CH_2)_nSR^{10}$; $O(CH_2)_nSS(CH_2)_nOR^{10}$, $O(CH_2)_nC(O)OR^{10}$,

$NH(CH_2)_nR^{10}$; $NH(CH_2)_nNR^{10}$;$NH(CH_2)_nOR^{10}$, $NH(CH_2)_nSR^{10}$; $S(CH_2)_nR^{10}$, $S(CH_2)_nNR^{10}$,

$S(CH_2)_nOR^{10}$, $S(CH_2)_nSR^{10}$ $O(CH_2CH_2O)_mCH_2CH_2OR^{10}$; $O(CH_2CH_2O)_mCH_2CH_2NHR^{10}$,

$NH(CH_2CH_2NH)_mCH_2CH_2NHR^{10}$; Q-R^{10}, O-Q-R^{10} N-Q-R^{10}, S-Q-R^{10} or -O-. W^4 is O, CH_2,

NH, or S.

X^1, X^2, X^3, and X^4 are each, independently, O or S.

Y^1, Y^2, Y^3, and Y^4 are each, independently, OH, O^-, OR^8, S, Se, BH_3^-, H, NHR^9, $N(R^9)_2$

alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted.

Z^1, Z^2, and Z^3 are each independently O, CH_2, NH, or S. Z^4 is OH, $(CH_2)_nR^{10}$,

$(CH_2)_nNHR^{10}$, $(CH_2)_n OR^{10}$, $(CH_2)_n SR^{10}$; $O(CH_2)_nR^{10}$; $O(CH_2)_nOR^{10}$, $O(CH_2)_nNR^{10}$,

$O(CH_2)_nSR^{10}$, $O(CH_2)_nSS(CH_2)_nOR^{10}$, $O(CH_2)_nC(O)OR^{10}$; $NH(CH_2)_nR^{10}$; $NH(CH_2)_nNR^{10}$

;$NH(CH_2)_nOR^{10}$, $NH(CH_2)_nSR^{10}$; $S(CH_2)_nR^{10}$, $S(CH_2)_nNR^{10}$, $S(CH_2)_nOR^{10}$, $S(CH_2)_nSR^{10}$

$O(CH_2CH_2O)_mCH_2CH_2OR^{10}$, $O(CH_2CH_2O)_mCH_2CH_2NHR^{10}$,

$NH(CH_2CH_2NH)_mCH_2CH_2NHR^{10}$; $Q-R^{10}$, $O-Q-R^{10}$ $N-Q-R^{10}$, $S-Q-R^{10}$.

x is 5-100, chosen to comply with a length for an RNA agent described herein.

R^7 is H; or is together combined with R^4, R^5, or R^6 to form an [-O-CH₂-] covalently bound bridge between the sugar 2' and 4' carbons.

R^8 is alkyl, cycloalkyl, aryl, aralkyl, heterocyclyl, heteroaryl, amino acid, or sugar; R^9 is NH_2, alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, or amino acid; and R^{10} is H; fluorophore (pyrene, TAMRA, fluorescein, Cy3 or Cy5 dyes); sulfur, silicon, boron or ester protecting group; intercalating agents (e.g. acridines), cross-linkers (e.g. psoralene, mitomycin C), porphyrins (TPPC4,texaphyrin, Sapphyrin), polycyclic aromatic hydrocarbons (e.g., phenazine, dihydrophenazine), artificial endonucleases (e.g. EDTA), lipophilic carriers (cholesterol, cholic acid, adamantane acetic acid, 1-pyrene butyric acid, dihydrotestosterone, 1,3-Bis-O(hexadecyl)glycerol, geranyloxyhexyl group, hexadecylglycerol, borneol, menthol, 1,3-propanediol, heptadecyl group, palmitic acid,myristic acid,O3-(oleoyl)lithocholic acid, O3-(oleoyl)cholenic acid, dimethoxytrityl, or phenoxazine)and peptide conjugates (e.g., antennapedia peptide, Tat peptide), alkylating agents, phosphate, amino, mercapto, PEG (e.g., PEG-40K), MPEG, [MPEG]₂, polyamino; alkyl, cycloalkyl, aryl, aralkyl, heteroaryl; radiolabelled markers, enzymes, haptens (e.g. biotin), transport/absorption facilitators (e.g., aspirin, vitamin E, folic acid), synthetic ribonucleases (e.g., imidazole, bisimidazole, histamine, imidazole clusters, acridine-imidazole conjugates, Eu3+ complexes of tetraazamacrocycles); or an RNA agent. m is 0-1,000,000, and n is 0-20. Q is a spacer selected from the group consisting of abasic sugar, amide, carboxy, oxyamine, oxyimine, thioether, disulfide, thiourea, sulfonamide, or morpholino, biotin or fluorescein reagents.

Preferred RNA agents in which the entire phosphate group has been replaced have the following structure (see Formula 3 below):
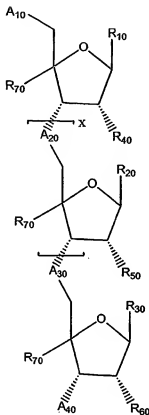
FORMULA 3

Referring to Formula 3, A^{10}-A^{40} is L-G-L; A^{10} and/or A^{40} may be absent, in which L is a linker, wherein one or both L may be present or absent and is selected from the group consisting of $CH_2(CH_2)_g$; $N(CH_2)_g$; $O(CH_2)_g$; $S(CH_2)_g$. G is a functional group selected from the group consisting of siloxane, carbonate, carboxymethyl, carbamate, amide, thioether, ethylene oxide linker, sulfonate, sulfonamide, thioformacetal, formacetal, oxime, methyleneimino, methylenemethylimino, methylenehydrazo, methylenedimethylhydrazo and methyleneoxymethylimino.

R^{10}, R^{20}, and R^{30} are each, independently, H, (i.e. abasic nucleotides), adenine, guanine, cytosine and uracil, inosine, thymine, xanthine, hypoxanthine, nubularine, tubercidine, isoguanisine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 5-halouracil, 5-(2-aminopropyl)uracil, 5-amino allyl uracil, 8-halo, amino, thiol, thioalkyl, hydroxyl and other 8-substituted adenines and guanines, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine, 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine, dihydrouracil, 3-deaza-5-azacytosine, 2-aminopurine, 5-alkyluracil, 7-alkylguanine, 5-alkyl cytosine, 7-deazaadenine, 7-deazaguanine, N6, N6-dimethyladenine, 2,6-diaminopurine, 5-amino-allyl-uracil, N3-methyluracil substituted 1,2,4-triazoles, 2-pyridinone, 5-nitroindole, 3-nitropyrrole, 5-methoxyuracil, uracil-5-oxyacetic acid, 5-methoxycarbonylmethyluracil, 5-methyl-2-thiouracil, 5-methoxycarbonylmethyl-2-thiouracil, 5-methylaminomethyl-2-thiouracil, 3-(3-amino-3carboxypropyl)uracil, 3-methylcytosine, 5-methylcytosine, N^4-acetyl cytosine, 2-thiocytosine, N6-methyladenine, N6-isopentyladenine, 2-methylthio-N6-isopentenyladenine, N-methylguanines, or O-alkylated bases.

R^{40}, R^{50}, and R^{60} are each, independently, OR^8, $O(CH_2CH_2O)_mCH_2CH_2OR^8$; $O(CH_2)_nR^9$; $O(CH_2)_nOR^9$, H; halo; NH_2; NHR^8; $N(R^8)_2$; $NH(CH_2CH_2NH)_mCH_2CH_2R^9$; $NHC(O)R^8$;; cyano; mercapto, SR^7; alkyl-thio-alkyl; alkyl, aralkyl, cycloalkyl, aryl, heteroaryl, alkenyl, alkynyl, each of which may be optionally substituted with halo, hydroxy, oxo, nitro, haloalkyl, alkyl, alkaryl, aryl, aralkyl, alkoxy, aryloxy, amino, alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, acylamino, alkylcarbamoyl, arylcarbamoyl, aminoalkyl, alkoxycarbonyl, carboxy, hydroxyalkyl, alkanesulfonyl, alkanesulfonamido, arenesulfonamido, aralkylsulfonamido, alkylcarbonyl, acyloxy, cyano, and ureido groups; or R^{40}, R^{50}, or R^{60} together combine with R^{70} to form an [-O-CH$_2$-] covalently bound bridge between the sugar 2' and 4' carbons.

x is 5-100 or chosen to comply with a length for an RNA agent described herein.

R^{70} is H; or is together combined with R^{40}, R^{50}, or R^{60} to form an [-O-CH$_2$-] covalently bound bridge between the sugar 2' and 4' carbons.

R^8 is alkyl, cycloalkyl, aryl, aralkyl, heterocyclyl, heteroaryl, amino acid, or sugar; and R^9 is NH_2, alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, or amino acid. m is 0-1,000,000, n is 0-20, and g is 0-2.

Preferred nucleoside surrogates have the following structure (see Formula 4 below):

$$SLR^{100}\text{-}(M\text{-}SLR^{200})_x\text{-}M\text{-}SLR^{300}$$
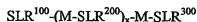
FORMULA 4

S is a nucleoside surrogate selected from the group consisting of mophilino, cyclobutyl, pyrrolidine and peptide nucleic acid. L is a linker and is selected from the group consisting of $CH_2(CH_2)_g$; $N(CH_2)_g$; $O(CH_2)_g$; $S(CH_2)_g$; $\text{-}C(O)(CH_2)_n\text{-}$ or may be absent. M is an amide bond; sulfonamide; sulfinate; phosphate group; modified phosphate group as described herein; or may be absent.

R^{100}, R^{200}, and R^{300} are each, independently, H (*i.e.*, abasic nucleotides), adenine, guanine, cytosine and uracil, inosine, thymine, xanthine, hypoxanthine, nubularine, tubercidine, isoguanisine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 5-halouracil, 5-(2-aminopropyl)uracil, 5-amino allyl uracil, 8-halo, amino, thiol, thioalkyl, hydroxyl and other 8-substituted adenines and guanines, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine, 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine, dihydrouracil, 3-deaza-5-azacytosine, 2-aminopurine, 5-alkyluracil, 7-alkylguanine, 5-alkyl cytosine, 7-deazaadenine, 7-deazaguanine, N6, N6-dimethyladenine, 2,6-diaminopurine, 5-amino-allyl-uracil, N3-methyluracil substituted 1, 2, 4,-triazoles, 2-pyridinones, 5-nitroindole, 3-nitropyrrole, 5-methoxyuracil, uracil-5-oxyacetic acid, 5-methoxycarbonylmethyluracil, 5-methyl-2-thiouracil, 5-methoxycarbonylmethyl-2-thiouracil, 5-methylaminomethyl-2-thiouracil, 3-(3-amino-3carboxypropyl)uracil, 3-methylcytosine, 5-methylcytosine, N^4-acetyl cytosine, 2-thiocytosine, N6-methyladenine, N6-isopentyladenine, 2-methylthio-N6-isopentenyladenine, N-methylguanines, or O-alkylated bases.

x is 5-100, or chosen to comply with a length for an RNA agent described herein; and g is 0-2.

Nuclease resistant monomers

An RNA, e.g., an iRNA agent, can incorporate a nuclease resistant monomer (NRM), such as those described herein and those described in copending, co-owned United States Provisional Application Serial No. 60/469,612, filed on May 9, 2003, and International Application No. PCT/US04/07070, both of which are hereby incorporated by reference.

In addition, the invention includes iRNA agents having an NRM and another element described herein. E.g., the invention includes an iRNA agent described herein, e.g., a palindromic iRNA agent, an iRNA agent having a non canonical pairing, an iRNA agent which targets a gene described herein, e.g., a gene active in the kidney, an iRNA agent having an architecture or structure described herein, an iRNA associated with an amphipathic delivery agent described herein, an iRNA associated with a drug delivery module described herein, an iRNA agent administered as described herein, or an iRNA agent formulated as described herein, which also incorporates an NRM.

An iRNA agent can include monomers which have been modifed so as to inhibit degradation, e.g., by nucleases, e.g., endonucleases or exonucleases, found in the body of a subject. These monomers are referred to herein as NRMs, or nuclease resistance promoting monomers or modifications. In many cases these modifications will modulate other properties of the iRNA agent as well, e.g., the ability to interact with a protein, e.g., a transport protein, e.g., serum albumin, or a member of the RISC (RNA-induced Silencing Complex), or the ability of the first and second sequences to form a duplex with one another or to form a duplex with another sequence, e.g., a target molecule.

While not wishing to be bound by theory, it is believed that modifications of the sugar, base, and/or phosphate backbone in an iRNA agent can enhance endonuclease and exonuclease resistance, and can enhance interactions with transporter proteins and one or more of the functional components of the RISC complex. Preferred modifications are those that increase exonuclease and endonuclease resistance and thus prolong the half-life of the iRNA agent prior to interaction with the RISC complex, but at the same time do not render the iRNA agent resistant to endonuclease activity in the RISC complex. Again, while not wishing to be bound by any theory, it is believed that placement of the modifications at or near the 3' and/or 5' end of

antisense strands can result in iRNA agents that meet the preferred nuclease resistance criteria delineated above. Again, still while not wishing to be bound by any theory, it is believed that placement of the modifications at e.g., the middle of a sense strand can result in iRNA agents that are relatively less likely to undergo off-targeting.

5   Modifications described herein can be incorporated into any double-stranded RNA and RNA-like molecule described herein, e.g., an iRNA agent. An iRNA agent may include a duplex comprising a hybridized sense and antisense strand, in which the antisense strand and/or the sense strand may include one or more of the modifications described herein. The anti sense strand may include modifications at the 3' end and/or the 5' end and/or at one or more positions

10   that occur 1-6 (e.g., 1-5, 1-4, 1-3, 1-2) nucleotides from either end of the strand. The sense strand may include modifications at the 3' end and/or the 5' end and/or at any one of the intervening positions between the two ends of the strand. The iRNA agent may also include a duplex comprising two hybridized antisense strands. The first and/or the second antisense strand may include one or more of the modifications described herein. Thus, one and/or both antisense

15   strands may include modifications at the 3' end and/or the 5' end and/or at one or more positions that occur 1-6 (e.g., 1-5, 1-4, 1-3, 1-2) nucleotides from either end of the strand. Particular configurations are discussed below.

        Modifications that can be useful for producing iRNA agents that meet the preferred nuclease resistance criteria delineated above can include one or more of the following chemical

20   and/or stereochemical modifications of the sugar, base, and/or phosphate backbone:

        (i) chiral (S_P) thioates. Thus, preferred NRMs include nucleotide dimers with an enriched or pure for a particular chiral form of a modified phosphate group containing a heteroatom at the nonbridging position, e.g., Sp or Rp, at the position X, where this is the position normally occupied by the oxygen. The atom at X can also be S, Se, Nr$_2$, or Br$_3$. When X is S, enriched or

25   chirally pure Sp linkage is preferred. Enriched means at least 70, 80, 90, 95, or 99% of the preferred form. Such NRMs are discussed in more detail below;

        (ii) attachment of one or more cationic groups to the sugar, base, and/or the phosphorus atom of a phosphate or modified phosphate backbone moiety. Thus, preferred NRMs include monomers at the terminal position derivatized at a cationic group. As the 5' end of an antisense

30   sequence should have a terminal –OH or phosphate group this NRM is preferably not used at the 5' end of an anti-sense sequence. The group should be attached at a position on the base which minimizes interference with H bond formation and hybridization, e.g., away form the face which

interacts with the complementary base on the other strand, e.g., at the 5' position of a pyrimidine
or a 7-position of a purine. These are discussed in more detail below;

(iii) nonphosphate linkages at the termini. Thus, preferred NRMs include Non-phosphate
linkages, e.g., a linkage of 4 atoms which confers greater resistance to cleavage than does a
phosphate bond. Examples include 3' CH2-NCH₃-O-CH2-5' and 3' CH2-NH-(O=)-CH2-5'.;

(iv) 3'-bridging thiophosphates and 5'-bridging thiophosphates. Thus, preferred NRM's
can included these structures;

(v) L-RNA, 2'-5' linkages, inverted linkages, a-nucleosides. Thus, other preferred
NRM's include: L nucleosides and dimeric nucleotides derived from L-nucleosides; 2'-5'
phosphate, non-phosphate and modified phosphate linkages (e.g., thiophosphates,
phosphoramidates and boronophosphates); dimers having inverted linkages, e.g., 3'-3' or 5'-5'
linkages; monomers having an alpha linkage at the 1' site on the sugar, e.g., the structures
described herein having an alpha linkage;

(vi) conjugate groups. Thus, preferred NRM's can include e.g., a targeting moiety or a
conjugated ligand described herein conjugated with the monomer, e.g., through the sugar , base,
or backbone;

(vi) abasic linkages. Thus, preferred NRM's can include an abasic monomer, e.g., an
abasic monomer as described herein (e.g., a nucleobaseless monomer); an aromatic or
heterocyclic or polyheterocyclic aromatic monomer as described herein.; and

(vii) 5'-phosphonates and 5'-phosphate prodrugs. Thus, preferred NRM's include
monomers, preferably at the terminal position, e.g., the 5' position, in which one or more atoms
of the phosphate group is derivatized with a protecting group, which protecting group or groups,
are removed as a result of the action of a component in the subject's body, e.g, a carboxyesterase
or an enzyme present in the subject's body. E.g., a phosphate prodrug in which a carboxy
esterase cleaves the protected molecule resulting in the production of a thioate anion which
attacks a carbon adjacent to the O of a phosphate and resulting in the production of an
unprotected phosphate.

One or more different NRM modifications can be introduced into an iRNA agent or into
a sequence of an iRNA agent. An NRM modification can be used more than once in a sequence
or in an iRNA agent. As some NRM's interfere with hybridization the total number

incorporated, should be such that acceptable levels of iRNA agent duplex formation are maintained.

In some embodiments NRM modifications are introduced into the terminal the cleavage site or in the cleavage region of a sequence (a sense strand or sequence) which does not target a desired sequence or gene in the subject. This can reduce off-target silencing.

Chiral S_P Thioates

A modification can include the alteration, *e.g.*, replacement, of one or both of the non-linking (X and Y) phosphate oxygens and/or of one or more of the linking (W and Z) phosphate oxygens. Formula X below depicts a phosphate moiety linking two sugar/sugar surrogate-base moieties, SB$_1$ and SB$_2$.
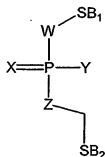
FORMULA X

In certain embodiments, one of the non-linking phosphate oxygens in the phosphate backbone moiety (X and Y) can be replaced by any one of the following: S, Se, BR$_3$ (R is hydrogen, alkyl, aryl, etc.), C (i.e., an alkyl group, an aryl group, etc.), H, NR$_2$ (R is hydrogen, alkyl, aryl, etc.), or OR (R is alkyl or aryl). The phosphorus atom in an unmodified phosphate group is achiral. However, replacement of one of the non-linking oxygens with one of the above atoms or groups of atoms renders the phosphorus atom chiral; in other words a phosphorus atom in a phosphate group modified in this way is a stereogenic center. The stereogenic phosphorus atom can possess either the "R" configuration (herein R$_P$) or the "S" configuration (herein S$_P$).

Thus if 60% of a population of stereogenic phosphorus atoms have the R_P configuration, then the remaining 40% of the population of stereogenic phosphorus atoms have the S_P configuration.

In some embodiments, iRNA agents, having phosphate groups in which a phosphate non-linking oxygen has been replaced by another atom or group of atoms, may contain a population of stereogenic phosphorus atoms in which at least about 50% of these atoms (e.g., at least about 60% of these atoms, at least about 70% of these atoms, at least about 80% of these atoms, at least about 90% of these atoms, at least about 95% of these atoms, at least about 98% of these atoms, at least about 99% of these atoms) have the S_P configuration. Alternatively, iRNA agents having phosphate groups in which a phosphate non-linking oxygen has been replaced by another atom or group of atoms may contain a population of stereogenic phosphorus atoms in which at least about 50% of these atoms (e.g., at least about 60% of these atoms, at least about 70% of these atoms, at least about 80% of these atoms, at least about 90% of these atoms, at least about 95% of these atoms, at least about 98% of these atoms, at least about 99% of these atoms) have the R_P configuration. In other embodiments, the population of stereogenic phosphorus atoms may have the S_P configuration and may be substantially free of stereogenic phosphorus atoms having the R_P configuration. In still other embodiments, the population of stereogenic phosphorus atoms may have the R_P configuration and may be substantially free of stereogenic phosphorus atoms having the S_P configuration. As used herein, the phrase "substantially free of stereogenic phosphorus atoms having the R_P configuration" means that moieties containing stereogenic phosphorus atoms having the R_P configuration cannot be detected by conventional methods known in the art (chiral HPLC, ^{1}H NMR analysis using chiral shift reagents, etc.). As used herein, the phrase "substantially free of stereogenic phosphorus atoms having the S_P configuration" means that moieties containing stereogenic phosphorus atoms having the S_P configuration cannot be detected by conventional methods known in the art (chiral HPLC, ^{1}H NMR analysis using chiral shift reagents, etc.).

In a preferred embodiment, modified iRNA agents contain a phosphorothioate group, i.e., a phosphate groups in which a phosphate non-linking oxygen has been replaced by a sulfur atom. In an especially preferred embodiment, the population of phosphorothioate stereogenic phosphorus atoms may have the S_P configuration and be substantially free of stereogenic phosphorus atoms having the R_P configuration.

Phosphorothioates may be incorporated into iRNA agents using dimers e.g., formulas X-1 and X-2. The former can be used to introduce phosphorothioate

56
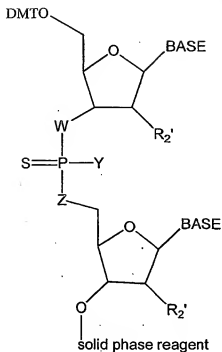
X-1
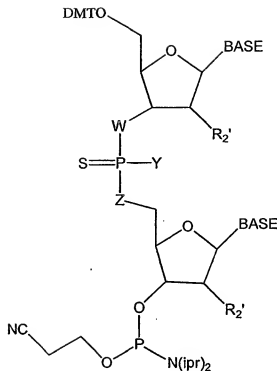
X-2

at the 3' end of a strand, while the latter can be used to introduce this modification at the 5' end or at a position that occurs e.g., 1, 2, 3, 4, 5, or 6 nucleotides from either end of the strand. In the above formulas, Y can be 2-cyanoethoxy, W and Z can be O, R_2, can be, e.g., a substituent that can impart the C-3 endo configuration to the sugar (e.g., OH, F, OCH_3), DMT is dimethoxytrityl, and "BASE" can be a natural, unusual, or a universal base.

X-1 and X-2 can be prepared using chiral reagents or directing groups that can result in phosphorothioate-containing dimers having a population of stereogenic phosphorus atoms having essentially only the R_P configuration (i.e., being substantially free of the S_P configuration) or only the S_P configuration (i.e., being substantially free of the R_P configuration). Alternatively, dimers can be prepared having a population of stereogenic phosphorus atoms in which about 50% of the atoms have the R_P configuration and about 50% of the atoms have the S_P configuration. Dimers having stereogenic phosphorus atoms with the R_P configuration can be

identified and separated from dimers having stereogenic phosphorus atoms with the S_P configuration using e.g., enzymatic degradation and/or conventional chromatography techniques.

Cationic Groups

Modifications can also include attachment of one or more cationic groups to the sugar, base, and/or the phosphorus atom of a phosphate or modified phosphate backbone moiety. A cationic group can be attached to any atom capable of substitution on a natural, unusual or universal base. A preferred position is one that does not interfere with hybridization, i.e., does not interfere with the hydrogen bonding interactions needed for base pairing. A cationic group can be attached e.g., through the C2' position of a sugar or analogous position in a cyclic or acyclic sugar surrogate. Cationic groups can include e.g., protonated amino groups, derived from e.g., O-AMINE (AMINE = NH_2; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamino); aminoalkoxy, e.g., $O(CH_2)_n$AMINE, (e.g., AMINE = NH_2; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamino); amino (e.g. NH_2; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, or amino acid); or $NH(CH_2CH_2NH)_nCH_2CH_2$-AMINE (AMINE = NH_2; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino).

Nonphosphate Linkages

Modifications can also include the incorporation of nonphosphate linkages at the 5' and/or 3' end of a strand. Examples of nonphosphate linkages which can replace the phosphate group include methyl phosphonate, hydroxylamino, siloxane, carbonate, carboxymethyl, carbamate, amide, thioether, ethylene oxide linker, sulfonate, sulfonamide, thioformacetal, formacetal, oxime, methyleneimino, methylenemethylimino, methylenehydrazo, methylenedimethylhydrazo and methyleneoxymethylimino. Preferred replacements include the methyl phosphonate and hydroxylamino groups.

3'-bridging thiophosphates and 5'-bridging thiophosphates; locked-RNA, 2'-5' likages, inverted linkages, α-nucleosides; conjugate groups; abasic linkages; and 5'-phosphonates and 5'-phosphate prodrugs

Referring to formula **X** above, modifications can include replacement of one of the bridging or linking phosphate oxygens in the phosphate backbone moiety (W and Z). Unlike the

situation where only one of X or Y is altered, the phosphorus center in the phosphorodithioates is achiral which precludes the formation of iRNA agents containing a stereogenic phosphorus atom.

5   Modifications can also include linking two sugars via a phosphate or modified phosphate group through the 2' position of a first sugar and the 5' position of a second sugar. Also contemplated are inverted linkages in which both a first and second sugar are eached linked through the respective 3' positions. Modified RNA's can also include "abasic" sugars, which lack a nucleobase at C-1'. The sugar group can also contain one or more carbons that possess the opposite stereochemical configuration than that of the corresponding carbon in ribose. Thus, a

10   modified iRNA agent can include nucleotides containing e.g., arabinose, as the sugar. In another subset of this modification, the natural, unusual, or universal base may have the α-configuration. Modifications can also include L-RNA.

       Modifications can also include 5'-phosphonates, e.g., $P(O)(O^-)_2$-X-$C^{5'}$-sugar (X= CH2, CF2, CHF and 5'-phosphate prodrugs, e.g., $P(O)[OCH2CH2SC(O)R]_2CH_2C^{5'}$-sugar. In the

15   latter case, the prodrug groups may be decomposed via reaction first with carboxy esterases. The remaining ethyl thiolate group via intramolecular S_N2 displacement can depart as episulfide to afford the underivatized phosphate group.

       Modification can also include the addition of conjugating groups described elseqhere herein, which are prefereably attached to an iRNA agent through any amino group available for

20   conjugation.

       Nuclease resistant modifications include some which can be placed only at the terminus and others which can go at any position. Generally the modifications that can inhibit hybridization so it is preferably to use them only in terminal regions, and preferrable to not use them at the cleavage site or in the cleavage region of an sequence which targets a subject

25   sequence or gene.. The can be used anywhere in a sense sequence, provided that sufficient hybridization between the two sequences of the iRNA agent is maintained. In some embodiments it is desirabable to put the NRM at the cleavage site or in the cleavage region of a sequence which does not target a subject sequence or gene,as it can minimize off-target silencing.

30   In addition, an iRNA agent described herein can have an overhang which does not form a duplex structure with the other sequence of the iRNA agent—it is an overhang, but it does

hybridize, either with itself, or with another nucleic acid, other than the other sequence of the iRNA agent.

In most cases, the nuclease-resistance promoting modifications will be distributed differently depending on whether the sequence will target a sequence in the subject (often referred to as an anti-sense sequence) or will not target a sequence in the subject (often referred to as a sense sequence). If a sequence is to target a sequence in the subject, modifications which interfer with or inhibit endonuclease cleavage should not be inserted in the region which is subject to RISC mediated cleavage, e.g., the cleavage site or the cleavage region (As described in Elbashir *et al.*, 2001, Genes and Dev. 15: 188, hereby incorporated by reference, cleavage of the target occurs about in the middle of a 20 or 21 nt guide RNA, or about 10 or 11 nucleotides upstream of the first nucleotide which is complementary to the guide sequence. As used herein cleavage site refers to the nucleotide on either side of the cleavage site, on the target or on the iRNA agent strand which hybridizes to it. Cleavage region means an nucleotide with 1, 2, or 3 nucletides of the cleave site, in either direction.)

Such modifications can be introduced into the terminal regions, e.g., at the terminal position or with .2, 3, 4, or 5 positions of the terminus, of a sequence which targets or a sequence which does not target a sequence in the subject.

An iRNA agent can have a first and a second strand chosen from the following:

a first strand which does not target a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 3' end;

a first strand which does not target a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 5' end;

a first strand which does not target a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 3' end and which has a NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 5' end;

a first strand which does not target a sequence and which has an NRM modification at the cleavage site or in the cleavage region;

a first strand which does not target a sequence and which has an NRM modification at the cleavage site or in the cleavage region and one or more of an NRM modification at or within 1,

2, 3, 4, 5 , or 6 positions from the 3' end, a NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 5' end, or NRM modifications at or within 1, 2, 3, 4, 5 , or 6 positions from both the 3' and the 5' end; and

a second strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 3' end;

a second strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 5' end (5' end NRM modifications are preferentially not at the terminus but rather at a position 1, 2, 3, 4, 5 , or 6 away from the 5' terminus of an antisense strand);

a second strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 3' end and which has a NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 5' end;

a second strand which targets a sequence and which preferably does not have an an NRM modification at the cleavage site or in the cleavage region;

a second strand which targets a sequence and which does not have an NRM modification at the cleavage site or in the cleavage region and one or more of an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 3' end, a NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 5' end, or NRM modifications at or within 1, 2, 3, 4, 5 , or 6 positions from both the 3' and the 5' end(5' end NRM modifications are preferentially not at the terminus but rather at a position 1, 2, 3, 4, 5 , or 6 away from the 5' terminus of an antisense strand).

An iRNA agent can also target two sequences and can have a first and second strand chosen from:

a first strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 3' end;

a first strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 5' end (5' end NRM modifications are preferentially not at the terminus but rather at a position 1, 2, 3, 4, 5 , or 6 away from the 5' terminus of an antisense strand);

a first strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 3' end and which has a NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 5' end;

a first strand which targets a sequence and which preferably does not have an an NRM modification at the cleavage site or in the cleavage region;

a first strand which targets a sequence and which dose not have an NRM modification at the cleavage site or in the cleavage region and one or more of an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 3' end, a NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 5' end, or NRM modifications at or within 1, 2, 3, 4, 5 , or 6 positions from both the 3' and the 5' end(5' end NRM modifications are preferentially not at the terminus but rather at a position 1, 2, 3, 4, 5 , or 6 away from the 5' terminus of an antisense strand) and

a second strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 3' end;

a second strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 5' end (5' end NRM modifications are preferentially not at the terminus but rather at a position 1, 2, 3, 4, 5 , or 6 away from the 5' terminus of an antisense strand);

a second strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 3' end and which has a NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 5' end;

a second strand which targets a sequence and which preferably does not have an an NRM modification at the cleavage site or in the cleavage region;

a second strand which targets a sequence and which dose not have an NRM modification at the cleavage site or in the cleavage region and one or more of an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 3' end, a NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 5' end, or NRM modifications at or within 1, 2, 3, 4, 5 , or 6 positions from both the 3' and the 5' end(5' end NRM modifications are preferentially not at the terminus but rather at a position 1, 2, 3, 4, 5 , or 6 away from the 5' terminus of an antisense strand).

Ribose Mimics

An RNA, e.g., an iRNA agent, can incorporate a ribose mimic, such as those described herein and those described in copending co-owned United States Provisional Application Serial No. 60/454,962, filed on March 13, 2003, and International Application No. PCT/US04/07070, both of which are hereby incorporated by reference.

In addition, the invention includes iRNA agents having a ribose mimic and another element described herein. E.g., the invention includes an iRNA agent described herein, e.g., a palindromic iRNA agent, an iRNA agent having a non canonical pairing, an iRNA agent which targets a gene described herein, e.g., a gene active in the kidney, an iRNA agent having an architecture or structure described herein, an iRNA associated with an amphipathic delivery agent described herein, an iRNA associated with a drug delivery module described herein, an iRNA agent administered as described herein, or an iRNA agent formulated as described herein, which also incorporates a ribose mimic.

Thus, an aspect of the invention features an iRNA agent that includes a secondary hydroxyl group, which can increase efficacy and/or confer nuclease resistance to the agent. Nucleases, e.g., cellular nucleases, can hydrolyze nucleic acid phosphodiester bonds, resulting in partial or complete degradation of the nucleic acid. The secondary hydroxy group confers nuclease resistance to an iRNA agent by rendering the iRNA agent less prone to nuclease degradation relative to an iRNA which lacks the modification. While not wishing to be bound by theory, it is believed that the presence of a secondary hydroxyl group on the iRNA agent can act as a structural mimic of a 3' ribose hydroxyl group, thereby causing it to be less susceptible to degradation.

The secondary hydroxyl group refers to an "OH" radical that is attached to a carbon atom substituted by two other carbons and a hydrogen. The secondary hydroxyl group that confers nuclease resistance as described above can be part of any acyclic carbon-containing group. The hydroxyl may also be part of any cyclic carbon-containing group, and preferably one or more of the following conditions is met (1) there is no ribose moiety between the hydroxyl group and the terminal phosphate group or (2) the hydroxyl group is not on a sugar moiety which is coupled to a base.. The hydroxyl group is located at least two bonds (e.g., at least three bonds away, at least four bonds away, at least five bonds away, at least six bonds away, at least seven bonds away, at least eight bonds away, at least nine bonds away, at least ten bonds away, etc.) from the terminal phosphate group phosphorus of the iRNA agent. In preferred embodiments, there are five

intervening bonds between the terminal phosphate group phosphorus and the secondary hydroxyl group.

Preferred iRNA agent delivery modules with five intervening bonds between the terminal phosphate group phosphorus and the secondary hydroxyl group have the following structure (see formula Y below):
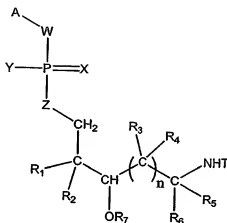
(Y)

Referring to formula Y, A is an iRNA agent, including any iRNA agent described herein. The iRNA agent may be connected directly or indirectly (e.g., through a spacer or linker) to "W" of the phosphate group. These spacers or linkers can include e.g., $-(CH_2)_n-$, $-(CH_2)_nN-$, $-(CH_2)_nO-$, $-(CH_2)_nS-$, $O(CH_2CH_2O)_nCH_2CH_2OH$ (e.g., n = 3 or 6), abasic sugars, amide, carboxy, amine, oxyamine, oxyimine, thioether, disulfide, thiourea, sulfonamide, or morpholino, or biotin and fluorescein reagents.

The iRNA agents can have a terminal phosphate group that is unmodified (e.g., W, X, Y, and Z are O) or modified. In a modified phosphate group, W and Z can be independently NH, O, or S; and X and Y can be independently S, Se, BH_3^-, C_1-C_6 alkyl, C_6-C_{10} aryl, H, O, O^-, alkoxy or amino (including alkylamino, arylamino, etc.). Preferably, W, X and Z are O and Y is S.

R_1 and R_3 are each, independently, hydrogen; or C_1-C_{100} alkyl, optionally substituted with hydroxyl, amino, halo, phosphate or sulfate and/or may be optionally inserted with N, O, S, alkenyl or alkynyl.

R_2 is hydrogen; C_1-C_{100} alkyl, optionally substituted with hydroxyl, amino, halo, phosphate or sulfate and/or may be optionally inserted with N, O, S, alkenyl or alkynyl; or, when n is 1, R_2 may be taken together with with R_4 or R_6 to form a ring of 5-12 atoms.

R_4 is hydrogen; C_1-C_{100} alkyl, optionally substituted with hydroxyl, amino, halo, phosphate or sulfate and/or may be optionally inserted with N, O, S, alkenyl or alkynyl; or, when n is 1, R_4 may be taken together with with R_2 or R_5 to form a ring of 5-12 atoms.

R_5 is hydrogen, C_1-C_{100} alkyl optionally substituted with hydroxyl, amino, halo, phosphate or sulfate and/or may be optionally inserted with N, O, S, alkenyl or alkynyl; or, when n is 1, R_5 may be taken together with with R_4 to form a ring of 5-12 atoms.

R_6 is hydrogen, C_1-C_{100} alkyl, optionally substituted with hydroxyl, amino, halo, phosphate or sulfate and/or may be optionally inserted with N, O, S, alkenyl or alkynyl, or, when n is 1, R_6 may be taken together with with R_2 to form a ring of 6-10 atoms;

R_7 is hydrogen, C_1-C_{100} alkyl, or $C(O)(CH_2)_qC(O)NHR_9$; T is hydrogen or a functional group; n and q are each independently 1-100; R_8 is C_1-C_{10} alkyl or C_6-C_{10} aryl; and R_9 is hydrogen, C1-C10 alkyl, C6-C10 aryl or a solid support agent.

Preferred embodiments may include one of more of the following subsets of iRNA agent delivery modules.

In one subset of RNAi agent delivery modules, A can be connected directly or indirectly through a terminal 3' or 5' ribose sugar carbon of the RNA agent.

In another subset of RNAi agent delivery modules, X, W, and Z are O and Y is S.

In still yet another subset of RNAi agent delivery modules, n is 1, and R_2 and R_6 are taken together to form a ring containing six atoms and R_4 and R_5 are taken together to form a ring containing six atoms. Preferably, the ring system is a *trans*-decalin. For example, the RNAi agent delivery module of this subset can include a compound of Formula (Y-1):
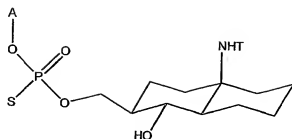
The functional group can be, for example, a targeting group (e.g., a steroid or a carbohydrate), a reporter group (e.g., a fluorophore), or a label (an isotopically labelled moiety). The targeting group can further include protein binding agents, endothelial cell targeting groups (e.g., RGD peptides and mimetics), cancer cell targeting groups (e.g., folate Vitamin B12, Biotin), bone cell targeting groups (e.g., bisphosphonates, polyglutamates, polyaspartates), multivalent mannose (for e.g., macrophage testing), lactose, galactose, N-acetyl-galactosamine, monoclonal antibodies, glycoproteins, lectins, melanotropin, or thyrotropin.

As can be appreciated by the skilled artisan, methods of synthesizing the compounds of the formulae herein will be evident to those of ordinary skill in the art. The synthesized compounds can be separated from a reaction mixture and further purified by a method such as column chromatography, high pressure liquid chromatography, or recrystallization. Additionally, the various synthetic steps may be performed in an alternate sequence or order to give the desired compounds. Synthetic chemistry transformations and protecting group methodologies (protection and deprotection) useful in synthesizing the compounds described herein are known in the art and include, for example, those such as described in R. Larock, *Comprehensive Organic Transformations*, VCH Publishers (1989); T.W. Greene and P.G.M. Wuts, *Protective Groups in Organic Synthesis*, 2d. Ed., John Wiley and Sons (1991); L. Fieser and M. Fieser, *Fieser and Fieser's Reagents for Organic Synthesis*, John Wiley and Sons (1994); and L. Paquette, ed., *Encyclopedia of Reagents for Organic Synthesis*, John Wiley and Sons (1995), and subsequent editions thereof.

Ribose Replacement Monomer Subunits

iRNA agents can be modified in a number of ways which can optimize one or more characteristics of the iRNA agent. An RNA agent, e.g., an iRNA agent can include a ribose replacement monomer subunit (RRMS), such as those described herein and those described in one or more United States Provisional Application Serial No. 60/493,986, filed on August 8, 2003, which is hereby incorporated by reference; United States Provisional Application Serial No. 60/494,597, filed on August 11, 2003, which is hereby incorporated by reference; United States Provisional Application Serial No. 60/506,341, filed on September 26, 2003, which is hereby incorporated by reference; United States Provisional Application Serial No. 60/158,453, filed on November 7, 2003, which is hereby incorporated by reference; and International Application No. PCT/US04/07070, filed March 8, 2004, which is hereby incorporated by reference.

In addition, the invention includes iRNA agents having a RRMS and another element described herein. E.g., the invention includes an iRNA agent described herein, e.g., a palindromic iRNA agent, an iRNA agent having a non canonical pairing, an iRNA agent which targets a gene described herein, e.g., a gene active in the kidney, an iRNA agent having an architecture or structure described herein, an iRNA associated with an amphipathic delivery agent described herein, an iRNA associated with a drug delivery module described herein, an iRNA agent administered as described herein, or an iRNA agent formulated as described herein, which also incorporates a RRMS.

The ribose sugar of one or more ribonucleotide subunits of an iRNA agent can be replaced with another moiety, e.g., a non-carbohydrate (preferably cyclic) carrier. A ribonucleotide subunit in which the ribose sugar of the subunit has been so replaced is referred to herein as a ribose replacement modification subunit (RRMS). A cyclic carrier may be a carbocyclic ring system, i.e., all ring atoms are carbon atoms, or a heterocyclic ring system, i.e., one or more ring atoms may be a heteroatom, e.g., nitrogen, oxygen, sulfur. The cyclic carrier may be a monocyclic ring system, or may contain two or more rings, e.g. fused rings. The cyclic carrier may be a fully saturated ring system, or it may contain one or more double bonds.

The carriers further include (i) at least two "backbone attachment points" and (ii) at least one "tethering attachment point." A "backbone attachment point" as used herein refers to a

functional group, e.g. a hydroxyl group, or generally, a bond available for, and that is suitable for incorporation of the carrier into the backbone, e.g., the phosphate, or modified phosphate, e.g., sulfur containing, backbone, of a ribonucleic acid. A "tethering attachment point" as used herein refers to a constituent ring atom of the cyclic carrier, e.g., a carbon atom or a heteroatom (distinct from an atom which provides a backbone attachment point), that connects a selected moiety. The moiety can be, e.g., a ligand, e.g., a targeting or delivery moiety, or a moiety which alters a physical property, e.g., lipophilicity, of an iRNA agent. Optionally, the selected moiety is connected by an intervening tether to the cyclic carrier. Thus, it will include a functional group, e.g., an amino group, or generally, provide a bond, that is suitable for incorporation or tethering of another chemical entity, e.g., a ligand to the constituent ring.

Incorporation of one or more RRMSs described herein into an RNA agent, e.g., an iRNA agent, particularly when tethered to an appropriate entity, can confer one or more new properties to the RNA agent and/or alter, enhance or modulate one or more existing properties in the RNA molecule. E.g., it can alter one or more of lipophilicity or nuclease resistance. Incorporation of one or more RRMSs described herein into an iRNA agent can, particularly when the RRMS is tethered to an appropriate entity, modulate, e.g., increase, binding affinity of an iRNA agent to a target mRNA, change the geometry of the duplex form of the iRNA agent, alter distribution or target the iRNA agent to a particular part of the body, or modify the interaction with nucleic acid binding proteins (e.g., during RISC formation and strand separation).

Accordingly, in one aspect, the invention features, an iRNA agent preferably comprising a first strand and a second strand, wherein at least one subunit having a formula (R-1) is incorporated into at least one of said strands.
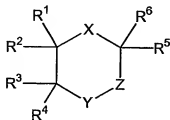
(R-1)

Referring to formula (R-1), X is $N(CO)R^7$, NR^7 or CH_2; Y is NR^8, O, S, CR^9R^{10}, or absent; and Z is $CR^{11}R^{12}$ or absent.

Each of R^1, R^2, R^3, R^4, R^9, and R^{10} is, independently, H, OR^a, OR^b, $(CH_2)_nOR^a$, or $(CH_2)_nOR^b$, provided that at least one of R^1, R^2, R^3, R^4, R^9, and R^{10} is OR^a or OR^b and that at least one of R^1, R^2, R^3, R^4, R^9, and R^{10} is $(CH_2)_nOR^a$, or $(CH_2)_nOR^b$ (when the RRMS is terminal, one of R^1, R^2, R^3, R^4, R^9, and R^{10} will include R^a and one will include R^b; when the RRMS is internal, two of R^1, R^2, R^3, R^4, R^9, and R^{10} will each include an R^b); further provided that preferably OR^a may only be present with $(CH_2)_nOR^b$ and $(CH_2)_nOR^a$ may only be present with OR^b.

Each of R^5, R^6, R^{11}, and R^{12} is, independently, H, C_1-C_6 alkyl optionally substituted with 1-3 R^{13}, or $C(O)NHR^7$; or R^5 and R^{11} together are C_3-C_8 cycloalkyl optionally substituted with R^{14}.

R^7 is C_1-C_{20} alkyl substituted with NR^cR^d; R^8 is C_1-C_6 alkyl; R^{13} is hydroxy, C_1-C_4 alkoxy, or halo; and R^{14} is NR^cR^7.

R^a is:

; and

R^b is:

$$A \atop \|$$
$$—P—O——Strand$$
$$\|$$
$$C$$
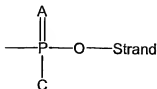
Each of A and C is, independently, O or S.

B is OH, O⁻, or

$$O \quad\quad O$$
$$\| \quad\quad\quad \|$$
$$O—P—O—P—OH$$
$$\| \quad\quad\quad \|$$
$$O⁻ \quad\quad O⁻$$
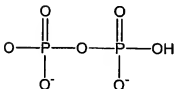
R^c is H or C1-C6 alkyl; R^d is H or a ligand; and n is 1-4.

In a preferred embodiment the ribose is replaced with a pyrroline scaffold, and X is $N(CO)R^7$ or NR^7, Y is CR^9R^{10}, and Z is absent.

In other preferred embodiments the ribose is replaced with a piperidine scaffold, and X is $N(CO)R^7$ or NR^7, Y is CR^9R^{10}, and Z is $CR^{11}R^{12}$.

In other preferred embodiments the ribose is replaced with a piperazine scaffold, and X is $N(CO)R^7$ or NR^7, Y is NR^8, and Z is $CR^{11}R^{12}$.

In other preferred embodiments the ribose is replaced with a morpholino scaffold, and X is $N(CO)R^7$ or NR^7, Y is O, and Z is $CR^{11}R^{12}$.

In other preferred embodiments the ribose is replaced with a decalin scaffold, and X isCH_2; Y is CR^9R^{10}; and Z is $CR^{11}R^{12}$; and R^5 and R^{11} together are C^6 cycloalkyl.

In other preferred embodiments the ribose is replaced with a decalin/indane scaffold and , and X is CH_2; Y is CR^9R^{10}; and Z is $CR^{11}R^{12}$; and R^5 and R^{11} together are C^5 cycloalkyl.

In other preferred embodiments, the ribose is replaced with a hydroxyproline scaffold.

RRMSs described herein may be incorporated into any double-stranded RNA-like molecule described herein, e.g., an iRNA agent. An iRNA agent may include a duplex comprising a hybridized sense and antisense strand, in which the antisense strand and/or the sense strand may include one or more of the RRMSs described herein. An RRMS can be introduced at one or more points in one or both strands of a double-stranded iRNA agent. An RRMS can be placed at or near (within 1, 2, or 3 positions) of the 3' or 5' end of the sense strand or at near (within 2 or 3 positions of) the 3' end of the antisense strand. In some embodiments it is preferred to not have an RRMS at or near (within 1, 2, or 3 positions of) the 5' end of the antisense strand. An RRMS can be internal, and will preferably be positioned in regions not critical for antisense binding to the target.

In an embodiment, an iRNA agent may have an RRMS at (or within 1, 2, or 3 positions of) the 3' end of the antisense strand. In an embodiment, an iRNA agent may have an RRMS at (or within 1, 2, or 3 positions of) the 3' end of the antisense strand and at (or within 1, 2, or 3 positions of) the 3' end of the sense strand. In an embodiment, an iRNA agent may have an RRMS at (or within 1, 2, or 3 positions of) the 3' end of the antisense strand and an RRMS at the 5' end of the sense strand, in which both ligands are located at the same end of the iRNA agent.

In certain embodiments, two ligands are tethered, preferably, one on each strand and are hydrophobic moieties. While not wishing to be bound by theory, it is believed that pairing of the hydrophobic ligands can stabilize the iRNA agent *via* intermolecular van der Waals interactions.

In an embodiment, an iRNA agent may have an RRMS at (or within 1, 2, or 3 positions of) the 3' end of the antisense strand and an RRMS at the 5' end of the sense strand, in which both RRMSs may share the same ligand (e.g., cholic acid) via connection of their individual tethers to separate positions on the ligand. A ligand shared between two proximal RRMSs is referred to herein as a "hairpin ligand."

In other embodiments, an iRNA agent may have an RRMS at the 3' end of the sense strand and an RRMS at an internal position of the sense strand. An iRNA agent may have an RRMS at an internal position of the sense strand; or may have an RRMS at an internal position

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of the antisense strand; or may have an RRMS at an internal position of the sense strand and an RRMS at an internal position of the antisense strand.

In preferred embodiments the iRNA agent includes a first and second sequences, which are preferably two separate molecules as opposed to two sequences located on the same strand, have sufficient complementarity to each other to hybridize (and thereby form a duplex region), e.g., under physiological conditions, e.g., under physiological conditions but not in contact with a helicase or other unwinding enzyme.

It is preferred that the first and second sequences be chosen such that the ds iRNA agent includes a single strand or unpaired region at one or both ends of the molecule. Thus, a ds iRNA agent contains first and second sequences, preferable paired to contain an overhang, e.g., one or two 5' or 3' overhangs but preferably a 3' overhang of 2-3 nucleotides. Most embodiments will have a 3' overhang. Preferred sRNA agents will have single-stranded overhangs, preferably 3' overhangs, of 1 or preferably 2 or 3 nucleotides in length at each end. The overhangs can be the result of one strand being longer than the other, or the result of two strands of the same length being staggered. 5' ends are preferably phosphorylated.

An RNA agent, e.g., an iRNA agent, containing a preferred, but nonlimiting RRMS is presented as formula (R-2) in FIG. 4. The carrier includes two "backbone attachment points" (hydroxyl groups), a "tethering attachment point," and a ligand, which is connected indirectly to the carrier via an intervening tether. The RRMS may be the 5' or 3' terminal subunit of the RNA molecule, i.e., one of the two "W" groups may be a hydroxyl group, and the other "W" group may be a chain of two or more unmodified or modified ribonucleotides. Alternatively, the RRMS may occupy an internal position, and both "W" groups may be one or more unmodified or modified ribonucleotides. More than one RRMS may be present in a RNA molecule, e.g., an iRNA agent.

The modified RNA molecule of formula (R-2) can be obtained using oligonucleotide synthetic methods known in the art. In a preferred embodiment, the modified RNA molecule of formula (II) can be prepared by incorporating one or more of the corresponding RRMS monomer compounds (RRMS monomers, see, e.g., A, B, and C in FIG 4) into a growing sense or antisense strand, utilizing, e.g., phosphoramidite or H-phosphonate coupling strategies.

The RRMS monomers generally include two differently functionalized hydroxyl groups (OFG^1 and OFG^2 above), which are linked to the carrier molecule (see A in FIG. 4), and a

tethering attachment point. As used herein, the term "functionalized hydroxyl group" means that
the hydroxyl proton has been replaced by another substituent. As shown in representative
structures **B** and **C**, one hydroxyl group (OFG^1) on the carrier is functionalized with a protecting
group (PG). The other hydroxyl group (OFG^2) can be functionalized with either (1) a liquid or
solid phase synthesis reagent (solid circle) directly or indirectly through a linker, L, as in
B, or (2) a phosphorus-containing moiety, e.g., a phosphoramidite as in C. The tethering
attachment point may be connected to a hydrogen atom, a tether, or a tethered ligand at the time
that the monomer is incorporated into the growing sense or antisense strand (see R in Scheme 1).
Thus, the tethered ligand can be, but need not be attached to the monomer at the time that the
monomer is incorporated into the growing strand. In certain embodiments, the tether, the ligand
or the tethered ligand may be linked to a "precursor" RRMS after a "precursor" RRMS monomer
has been incorporated into the strand.

The (OFG^1) protecting group may be selected as desired, e.g., from T.W. Greene and
P.G.M. Wuts, *Protective Groups in Organic Synthesis*, 2d. Ed., John Wiley and Sons (1991).
The protecting group is preferably stable under amidite synthesis conditions, storage conditions,
and oligonucleotide synthesis conditions. Hydroxyl groups, -OH, are nucleophilic groups (i.e.,
Lewis bases), which react through the oxygen with electrophiles (i.e., Lewis acids). Hydroxyl
groups in which the hydrogen has been replaced with a protecting group, e.g., a triarylmethyl
group or a trialkylsilyl group, are essentially unreactive as nucleophiles in displacement
reactions. Thus, the protected hydroxyl group is useful in preventing e.g., homocoupling of
compounds exemplified by structure C during oligonucleotide synthesis. A preferred protecting
group is the dimethoxytrityl group.

When the OFG^2 in **B** includes a linker, e.g., a long organic linker, connected to a soluble
or insoluble support reagent, solution or solid phase synthesis techniques can be employed to
build up a chain of natural and/or modified ribonucleotides once OFG^1 is deprotected and free to
react as a nucleophile with another nucleoside or monomer containing an electrophilic group
(e.g., an amidite group). Alternatively, a natural or modified ribonucleotide or
oligoribonucleotide chain can be coupled to monomer **C** *via* an amidite group or H-phosphonate
group at OFG^2. Subsequent to this operation, OFG^1 can be deblocked, and the restored
nucleophilic hydroxyl group can react with another nucleoside or monomer containing an
electrophilic group (see FIG. 1). R' can be substituted or unsubstituted alkyl or alkenyl. In
preferred embodiments, R' is methyl, allyl or 2-cyanoethyl. R'' may a C_1-C_{10} alkyl group,
preferably it is a branched group containing three or more carbons, e.g., isopropyl.

OFG^4 in **B** can be hydroxyl functionalized with a linker, which in turn contains a liquid or solid phase synthesis support reagent at the other linker terminus. The support reagent can be any support medium that can support the monomers described herein. The monomer can be attached to an insoluble support via a linker, L, which allows the monomer (and the growing chain) to be solubilized in the solvent in which the support is placed. The solubilized, yet immobilized, monomer can react with reagents in the surrounding solvent; unreacted reagents and soluble by-products can be readily washed away from the solid support to which the monomer or monomer-derived products is attached. Alternatively, the monomer can be attached to a soluble support moiety, e.g., polyethylene glycol (PEG) and liquid phase synthesis techniques can be used to build up the chain. Linker and support medium selection is within skill of the art. Generally the linker may be -C(O)(CH$_2$)$_4$C(O)-, or -C(O)(CH$_2$)$_q$S-, preferably, it is oxalyl, succinyl or thioglycolyl. Standard control pore glass solid phase synthesis supports can not be used in conjunction with fluoride labile 5' silyl protecting groups because the glass is degraded by fluoride with a significant reduction in the amount of full-length product. Fluoride-stable polystyrene based supports or PEG are preferred.

Preferred carriers have the general formula (R-3) provided below. (In that structure preferred backbone attachment points can be chosen from R^1 or R^2; R^3 or R^4; or R^9 and R^{10} if Y is CR^{9}R^{10} (two positions are chosen to give two backbone attachment points, e.g., R^1 and R^4, or R^4 and R^9. Preferred tethering attachment points include R^7; R^5 or R^6 when X is CH$_2$. The carriers are described below as an entity, which can be incorporated into a strand. Thus, it is understood that the structures also encompass the situations wherein one (in the case of a terminal position) or two (in the case of an internal position) of the attachment points, e.g., R^1 or R^2; R^3 or R^4; or R^9 or R^{10} (when Y is CR^{9}R^{10}), is connected to the phosphate, or modified phosphate, e.g., sulfur containing, backbone. E.g., one of the above-named R groups can be -CH2-, wherein one bond is connected to the carrier and one to a backbone atom, e.g., a linking oxygen or a central phosphorus atom.)
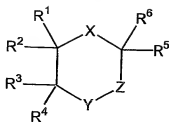
(R-3)

X is $N(CO)R^7$, NR^7 or CH_2; Y is NR^8, O, S, CR^9R^{10}; and Z is $CR^{11}R^{12}$ or absent.

Each of R^1, R^2, R^3, R^4, R^9, and R^{10} is, independently, H, OR^a, or $(CH_2)_nOR^b$, provided that at least two of R^1, R^2, R^3, R^4, R^9, and R^{10} are OR^a and/or $(CH_2)_nOR^b$.

Each of R^5, R^6, R^{11}, and R^{12} is, independently, a ligand, H, C_1-C_6 alkyl optionally substituted with 1-3 R^{13}, or $C(O)NHR^7$; or R^5 and R^{11} together are C_3-C_8 cycloalkyl optionally substituted with R^{14}.

R^7 is H, a ligand, or C_1-C_{20} alkyl substituted with NR^cR^4; R^8 is H or C_1-C_6 alkyl; R^{13} is hydroxy, C_1-C_4 alkoxy, or halo; R^{14} is NR^cR^7; R^{15} is C_1-C_6 alkyl optionally substituted with cyano, or C_2-C_6 alkenyl; R^{16} is C_1-C_{10} alkyl; and R^{17} is a liquid or solid phase support reagent.

L is $-C(O)(CH_2)_qC(O)-$, or $-C(O)(CH_2)_qS-$; R^a is CAr_3; R^b is $P(O)(O^-)H$, $P(OR^{15})N(R^{16})_2$ or $L-R^{17}$; R^c is H or C_1-C_6 alkyl; and R^d is H or a ligand.

Each Ar is, independently, C_6-C_{10} aryl optionally substituted with C_1-C_4 alkoxy; n is 1-4; and q is 0-4.

Exemplary carriers include those in which, e.g., X is $N(CO)R^7$ or NR^7, Y is CR^9R^{10}, and Z is absent; or X is $N(CO)R^7$ or NR^7, Y is CR^9R^{10}, and Z is $CR^{11}R^{12}$; or X is $N(CO)R^7$ or NR^7, Y is NR^8, and Z is $CR^{11}R^{12}$; or X is $N(CO)R^7$ or NR^7, Y is O, and Z is $CR^{11}R^{12}$; or X is CH_2; Y is CR^9R^{10}; Z is $CR^{11}R^{12}$, and R^5 and R^{11} together form C_6 cycloalkyl (**H**, z = 2), or the indane ring system, e.g., X is CH_2; Y is CR^9R^{10}; Z is $CR^{11}R^{12}$, and R^5 and R^{11} together form C_5 cycloalkyl (**H**, z = 1).

In certain embodiments, the carrier may be based on the pyrroline ring system or the 3-hydroxyproline ring system, e.g., X is $N(CO)R^7$ or NR^7, Y is CR^9R^{10}, and Z is absent (**D**). OFG^1 is preferably attached to a primary carbon, e.g., an exocyclic alkylene

D
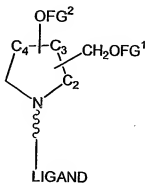
group, e.g., a methylene group, connected to one of the carbons in the five-membered ring ($-CH_2OFG^1$ in **D**). OFG^2 is preferably attached directly to one of the carbons in the five-membered ring ($-OFG^2$ in **D**). For the pyrroline-based carriers, $-CH_2OFG^1$ may be attached to C-2 and OFG^2 may be attached to C-3; or $-CH_2OFG^1$ may be attached to C-3 and OFG^2 may be attached to C-4.. . In certain embodiments, CH_2OFG^1 and OFG^2 may be geminally substituted to one of the above-referenced carbons.For the 3-hydroxyproline-based carriers, $-CH_2OFG^1$ may be attached to C-2 and OFG^2 may be attached to C-4. The pyrroline- and 3-hydroxyproline-based monomers may therefore contain linkages (e.g., carbon-carbon bonds) wherein bond rotation is restricted about that particular linkage, e.g. restriction resulting from the presence of a ring. Thus, CH_2OFG^1 and OFG^2 may be *cis* or *trans* with respect to one another in any of the pairings delineated above Accordingly, all *cis/trans* isomers are expressly included. The monomers may also contain one or more asymmetric centers and thus occur as racemates and racemic mixtures, single enantiomers, individual diastereomers and diastereomeric mixtures. All such isomeric forms of the monomers are expressly included. The tethering attachment point is preferably nitrogen.

In certain embodiments, the carrier may be based on the piperidine ring system (**E**), e.g., X is $N(CO)R^7$ or NR^7, Y is CR^9R^{10}, and Z is $CR^{11}R^{12}$. OFG^1 is preferably

E
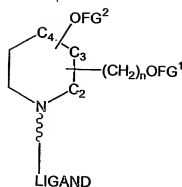
attached to a primary carbon, e.g., an exocyclic alkylene group, e.g., a methylene group (n=1) or ethylene group (n=2), connected to one of the carbons in the six-membered ring [-(CH$_2$)$_n$OFG^1 in E]. OFG^2 is preferably attached directly to one of the carbons in the six-membered ring (-OFG^2 in E). -(CH$_2$)$_n$OFG^1 and OFG^2 may be disposed in a geminal manner on the ring, i.e., both groups may be attached to the same carbon, e.g., at C-2, C-3, or C-4. Alternatively, -(CH$_2$)$_n$OFG^1 and OFG^2 may be disposed in a vicinal manner on the ring, i.e., both groups may be attached to adjacent ring carbon atoms, e.g., -(CH$_2$)$_n$OFG^1 may be attached to C-2 and OFG^2 may be attached to C-3; -(CH$_2$)$_n$OFG^1 may be attached to C-3 and OFG^2 may be attached to C-2; -(CH$_2$)$_n$OFG^1 may be attached to C-3 and OFG^2 may be attached to C-4; or -(CH$_2$)$_n$OFG^1 may be attached to C-4 and OFG^2 may be attached to C-3. The piperidine-based monomers may therefore contain linkages (e.g., carbon-carbon bonds) wherein bond rotation is restricted about that particular linkage, e.g. restriction resulting from the presence of a ring. Thus, -(CH$_2$)$_n$OFG^1 and OFG^2 may be *cis* or *trans* with respect to one another in any of the pairings delineated above. Accordingly, all *cis/trans* isomers are expressly included. The monomers may also contain one or more asymmetric centers and thus occur as racemates and racemic mixtures, single enantiomers, individual diastereomers and diastereomeric mixtures. All such isomeric forms of the monomers are expressly included. The tethering attachment point is preferably nitrogen.

In certain embodiments, the carrier may be based on the piperazine ring system (F), e.g., X is N(CO)R^7 or NR^7, Y is NR^8, and Z is CR^{11}R^{12}, or the morpholine ring system (G), e.g., X is N(CO)R^7 or NR^7, Y is O, and Z is CR^{11}R^{12}. OFG^1 is preferably
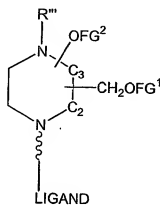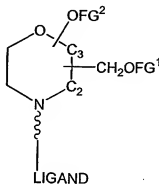

F

G

attached to a primary carbon, e.g., an exocyclic alkylene group, e.g., a methylene group, connected to one of the carbons in the six-membered ring (-CH$_2$OFG^1 in F or G). OFG^2 is preferably attached directly to one of the carbons in the six-membered rings (-OFG^2 in F or G). For both F and G, -CH$_2$OFG^1 may be attached to C-2 and OFG^2 may be attached to C-3; or *vice versa*. In certain embodiments, CH$_2$OFG^1 and OFG^2 may be geminally substituted to one of the above-referenced carbons. The piperazine- and morpholine-based monomers may therefore contain linkages (e.g., carbon-carbon bonds) wherein bond rotation is restricted about that particular linkage, e.g. restriction resulting from the presence of a ring. Thus, CH$_2$OFG^1 and OFG^2 may be *cis* or *trans* with respect to one another in any of the pairings delineated above. Accordingly, all *cis/trans* isomers are expressly included. The monomers may also contain one or more asymmetric centers and thus occur as racemates and racemic mixtures, single enantiomers, individual diastereomers and diastereomeric mixtures. All such isomeric forms of the monomers are expressly included. R''' can be, e.g., C$_1$-C$_6$ alkyl, preferably CH$_3$. The tethering attachment point is preferably nitrogen in both F and G.

In certain embodiments, the carrier may be based on the decalin ring system, e.g., X is CH$_2$; Y is CR^{9}R^{10}; Z is CR^{11}R^{12}, and R^5 and R^{11} together form C$_6$ cycloalkyl (H, z = 2), or the indane ring system, e.g., X is CH$_2$; Y is CR^{9}R^{10}; Z is CR^{11}R^{12}, and R^5 and R^{11} together form C$_5$ cycloalkyl (H, z = 1). OFG^1 is preferably attached to a primary carbon,

78

H
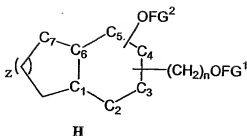
e.g., an exocyclic methylene group (n=1) or ethylene group (n=2) connected to one of C-2, C-3, C-4, or C-5 [-$(CH_2)_nOFG^1$ in H]. OFG^2 is preferably attached directly to one of C-2, C-3, C-4, or C-5 (-OFG^2 in H). -$(CH_2)_nOFG^1$ and OFG^2 may be disposed in a geminal manner on the ring,

i.e., both groups may be attached to the same carbon, e.g., at C-2, C-3, C-4, or C-5. Alternatively, -$(CH_2)_nOFG^1$ and OFG^2 may be disposed in a vicinal manner on the ring, i.e., both groups may be attached to adjacent ring carbon atoms, e.g., -$(CH_2)_nOFG^1$ may be attached to C-2 and OFG^2 may be attached to C-3; -$(CH_2)_nOFG^1$ may be attached to C-3 and OFG^2 may be attached to C-2; -$(CH_2)_nOFG^1$ may be attached to C-3 and OFG^2 may be attached to C-4; or -

$(CH_2)_nOFG^1$ may be attached to C-4 and OFG^2 may be attached to C-3; -$(CH_2)_nOFG^1$ may be attached to C-4 and OFG^2 may be attached to C-5; or -$(CH_2)_nOFG^1$ may be attached to C-5 and OFG^2 may be attached to C-4. The decalin-based monomers may therefore contain linkages (e.g., carbon-carbon bonds) wherein bond rotation is restricted about that particular linkage, e.g. restriction resulting from the presence of a ring. Thus, -$(CH_2)_nOFG^1$ and OFG^2 may

be *cis* or *trans* with respect to one another in any of the pairings delineated above. Accordingly, all *cis/trans* isomers are expressly included. The monomers may also contain one or more asymmetric centers and thus occur as racemates and racemic mixtures, single enantiomers, individual diastereomers and diastereomeric mixtures. All such isomeric forms of the monomers are expressly included. In a preferred embodiment, the substituents at C-1 and C-6 are *trans*

with respect to one another. The tethering attachment point is preferably C-6 or C-7.

Other carriers may include those based on 3-hydroxyproline (**J**). Thus, -$(CH_2)_nOFG^1$ and OFG^2 may be *cis* or *trans* with respect to one another. Accordingly, all *cis/trans* isomers are expressly included. The monomers may also contain one or more asymmetric centers

$$^2GFO(CH_2)_n\text{—}\underset{N}{\bigcirc}\text{—}OFG_1$$

LIGAND

J
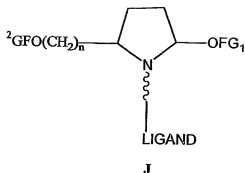
and thus occur as racemates and racemic mixtures, single enantiomers, individual diastereomers and diastereomeric mixtures. All such isomeric forms of the monomers are expressly included. The tethering attachment point is preferably nitrogen.

5   Representative carriers are shown in FIG. 5.

In certain embodiments, a moiety, e.g., a ligand may be connected indirectly to the carrier *via* the intermediacy of an intervening tether. Tethers are connected to the carrier at the tethering attachment point (TAP) and may include any C_1-C_{100} carbon-containing moiety, (e.g. C_1-C_{75}, C_1-C_{50}, C_1-C_{20}, C_1-C_{10}, C_1-C_6), preferably having at least one nitrogen atom. In preferred

10   embodiments, the nitrogen atom forms part of a terminal amino group on the tether, which may serve as a connection point for the ligand. Preferred tethers (underlined) include TAP-(CH_2)_nNH_2; TAP-C(O)(CH_2)_nNH_2; or TAP-NR''''(CH_2)_nNH_2, in which n is 1-6 and R'''' is C_1-C_6 alkyl. and R^d is hydrogen or a ligand. In other embodiments, the nitrogen may form part of a terminal oxyamino group, e.g., -ONH_2, or hydrazino group, -NHNH_2. The tether may optionally

15   be substituted, e.g., with hydroxy, alkoxy, perhaloalkyl, and/or optionally inserted with one or more additional heteroatoms, e.g., N, O, or S. Preferred tethered ligands may include, e.g., TAP-(CH_2)_nNH(LIGAND),

TAP-C(O)(CH_2)_nNH(LIGAND), or TAP-NR''''(CH_2)_nNH(LIGAND);

TAP-(CH_2)_nONH(LIGAND), TAP-C(O)(CH_2)_nONH(LIGAND), or

20   TAP-NR''''(CH_2)_nONH(LIGAND); TAP-(CH_2)_nNHNH_2(LIGAND),

TAP-C(O)(CH_2)_nNHNH_2(LIGAND), or TAP-NR''''(CH_2)_nNHNH_2(LIGAND).

In other embodiments the tether may include an electrophilic moiety, preferably at the terminal position of the tether. Preferred electrophilic moieties include, e.g., an aldehyde, alkyl halide, mesylate, tosylate, nosylate, or brosylate, or an activated carboxylic acid ester, e.g. an NHS ester, or a pentafluorophenyl ester. Preferred tethers (underlined) include TAP-$(CH_2)_nCHO$; TAP-$C(O)(CH_2)_nCHO$; or TAP-$NR''''(CH_2)_nCHO$, in which n is 1-6 and R'''' is C_1-C_6 alkyl; or TAP-$(CH_2)_nC(O)ONHS$; TAP-$C(O)(CH_2)_nC(O)ONHS$; or

TAP-$NR''''(CH_2)_nC(O)ONHS$, in which n is 1-6 and R'''' is C_1-C_6 alkyl;

TAP-$(CH_2)_nC(O)OC_6F_5$; TAP-$C(O)(CH_2)_nC(O)$ OC_6F_5; or TAP-$NR''''(CH_2)_nC(O)$ OC_6F_5, in which n is 1-6 and R'''' is C_1-C_6 alkyl; or -$(CH_2)_nCH_2LG$; TAP-$C(O)(CH_2)_nCH_2LG$; or TAP-$NR''''(CH_2)_nCH_2LG$, in which n is 1-6 and R'''' is C_1-C_6 alkyl (LG can be a leaving group, e.g., halide, mesylate, tosylate, nosylate, brosylate). Tethering can be carried out by coupling a nucleophilic group of a ligand, e.g., a thiol or amino group with an electrophilic group on the tether.

Tethered Entities

A wide variety of entities can be tethered to an iRNA agent, e.g., to the carrier of an RRMS. Examples are described below in the context of an RRMS but that is only preferred, entities can be coupled at other points to an iRNA agent. Preferred entities are those which target to the kidney, and also those that specifically target to tissues other than the kidney.

Preferred moieties are ligands, which are coupled, preferably covalently, either directly or indirectly via an intervening tether, to the RRMS carrier. In preferred embodiments, the ligand is attached to the carrier *via* an intervening tether. As discussed above, the ligand or tethered ligand may be present on the RRMS monomer when the RRMS monomer is incorporated into the growing strand. In some embodiments, the ligand may be incorporated into a "precursor" RRMS after a "precursor" RRMS monomer has been incorporated into the growing strand. For example, an RRMS monomer having, e.g., an amino-terminated tether (i.e., having no associated ligand), e.g., TAP-$(CH_2)_nNH_2$ may be incorporated into a growing sense or antisense strand. In a subsequent operation, i.e., after incorporation of the precursor monomer into the strand, a ligand having an electrophilic group, e.g., a pentafluorophenyl ester or aldehyde group, can subsequently be attached to the precursor RRMS by coupling the electrophilic group of the ligand with the terminal nucleophilic group of the precursor RRMS tether.

In preferred embodiments, a ligand alters the distribution, targeting or lifetime of an iRNA agent into which it is incorporated. In preferred embodiments a ligand provides an enhanced affinity for a selected target, e.g, molecule, cell or cell type, compartment, e.g., a cellular or organ compartment, tissue, organ or region of the body, as, e.g., compared to a species absent such a ligand. Preferred ligands will not take part in duplex pairing in a duplexed nucleic acid.

Preferred ligands can improve transport, hybridization, and specificity properties and may also improve nuclease resistance of the resultant natural or modified oligoribonucleotide, or a polymeric molecule comprising any combination of monomers described herein and/or natural or modified ribonucleotides.

Ligands in general can include therapeutic modifiers, e.g., for enhancing uptake; diagnostic compounds or reporter groups e.g., for monitoring distribution; cross-linking agents; and nuclease-resistance conferring moieties. General examples include lipids, steroids, vitamins, sugars, proteins, peptides, polyamines, and peptide mimics.

Ligands can include a naturally occurring substance, such as a protein (e.g., human serum albumin (HSA), low-density lipoprotein (LDL), or globulin); carbohydrate (e.g., a dextran, pullulan, chitin, chitosan, inulin, cyclodextrin or hyaluronic acid); or a lipid. The ligand may also be a recombinant or synthetic molecule, such as a synthetic polymer, e.g., a synthetic polyamino acid. Examples of polyamino acids include polyamino acid is a polylysine (PLL), poly L-aspartic acid, poly L-glutamic acid, styrene-maleic acid anhydride copolymer, poly(L-lactide-co-glycolied) copolymer, divinyl ether-maleic anhydride copolymer, N-(2-hydroxypropyl)methacrylamide copolymer (HMPA), polyethylene glycol (PEG), polyvinyl alcohol (PVA), polyurethane, poly(2-ethylacryllic acid), N-isopropylacrylamide polymers, or polyphosphazine. Example of polyamines include: polyethylenimine, polylysine (PLL), spermine, spermidine, polyamine, pseudopeptide-polyamine, peptidomimetic polyamine, dendrimer polyamine, arginine, amidine, protamine, cationic lipid, cationic porphyrin, quaternary salt of a polyamine, or an alpha helical peptide.

Ligands can also include targeting groups, e.g., a cell or tissue targeting agent, e.g., a lectin, glycoprotein, lipid or protein, e.g., an antibody, that binds to a specified cell type such as a kidney cell. A targeting group can be a thyrotropin, melanotropin, lectin, glycoprotein, surfactant protein A, Mucin carbohydrate, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-gulucosamine multivalent mannose, multivalent fucose, glycosylated

polyaminoacids, multivalent galactose, transferrin, bisphosphonate, polyglutamate, polyaspartate, a lipid, cholesterol, a steroid, bile acid, folate, vitamin B12, biotin, or an RGD peptide or RGD peptide mimetic.

Other examples of ligands include dyes, intercalating agents (e.g. acridines), cross-linkers (e.g. psoralene, mitomycin C), porphyrins (TPPC4, texaphyrin, Sapphyrin), polycyclic aromatic hydrocarbons (e.g., phenazine, dihydrophenazine), artificial endonucleases (e.g. EDTA), lipophilic molecules, e.g, cholesterol, cholic acid, adamantane acetic acid, 1-pyrene butyric acid, dihydrotestosterone, 1,3-Bis-O(hexadecyl)glycerol, geranyloxyhexyl group, hexadecylglycerol, borneol, menthol, 1,3-propanediol, heptadecyl group, palmitic acid, myristic acid,O3- (oleoyl)lithocholic acid, O3-(oleoyl)cholenic acid, dimethoxytrityl, or phenoxazine)and peptide conjugates (e.g., antennapedia peptide, Tat peptide), alkylating agents, phosphate, amino, mercapto, PEG (e.g., PEG-40K), MPEG, [MPEG]₂, polyamino, alkyl, substituted alkyl, radiolabeled markers, enzymes, haptens (e.g. biotin), transport/absorption facilitators (e.g., aspirin, vitamin E, folic acid), synthetic ribonucleases (e.g., imidazole, bisimidazole, histamine, imidazole clusters, acridine-imidazole conjugates, Eu3+ complexes of tetraazamacrocycles), dinitrophenyl, HRP, or AP.

Ligands can be proteins, e.g., glycoproteins, or peptides, e.g., molecules having a specific affinity for a co-ligand, or antibodies e.g., an antibody, that binds to a specified cell type such as a cancer cell, endothelial cell, or bone cell. Ligands may also include hormones and hormone receptors. They can also include non-peptidic species, such as lipids, lectins, carbohydrates, vitamins, cofactors, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-gulucosamine multivalent mannose, or multivalent fucose. The ligand can be, for example, a lipopolysaccharide, an activator of p38 MAP kinase, or an activator of NF-κB.

The ligand can be a substance, e.g, a drug, which can increase the uptake of the iRNA agent into the cell, for example, by disrupting the cell's cytoskeleton, e.g., by disrupting the cell's microtubules, microfilaments, and/or intermediate filaments. The drug can be, for example, taxon, vincristine, vinblastine, cytochalasin, nocodazole, japlakinolide, latrunculin A, phalloidin, swinholide A, indanocine, or myoservin.

The ligand can increase the uptake of the iRNA agent into the cell by activating an inflammatory response, for example. Exemplary ligands that would have such an effect include tumor necrosis factor alpha (TNFalpha), interleukin-1 beta, or gamma interferon.

In one aspect, the ligand is a lipid or lipid-based molecule. Such a lipid or lipid-based molecule preferably binds a serum protein, e.g., human serum albumin (HSA). An HSA binding ligand allows for distribution of the conjugate to a target tissue, e.g., a non-kidney target tissue of the body. For example, the target tissue can be the liver, including parenchymal cells of the liver.

5   Other molecules that can bind HSA can also be used as ligands. For example, neproxin or aspirin can be used. A lipid or lipid-based ligand can (a) increase resistance to degradation of the conjugate, (b) increase targeting or transport into a target cell or cell membrane, and/or (c) can be used to adjust binding to a serum protein, e.g., HSA.

A lipid based ligand can be used to modulate, e.g., control the binding of the conjugate to

10   a target tissue. For example, a lipid or lipid-based ligand that binds to HSA more strongly will be less likely to be targeted to the kidney and therefore less likely to be cleared from the body. A lipid or lipid-based ligand that binds to HSA less strongly can be used to target the conjugate to the kidney.

In a preferred embodiment, the lipid based ligand binds HSA. Preferably, it binds HSA

15   with a sufficient affinity such that the conjugate will be preferably distributed to a non-kidney tissue. However, it is preferred that the affinity not be so strong that the HSA-ligand binding cannot be reversed.

In another preferred embodiment, the lipid based ligand binds HSA weakly or not at all, such that the conjugate will be preferably distributed to the kidney. Other moieties that target to

20   kidney cells can also be used in place of or in addition to the lipid based ligand.

In another aspect, the ligand is a moiety, e.g., a vitamin, which is taken up by a target cell, e.g., a proliferating cell. These are particularly useful for treating disorders characterized by unwanted cell proliferation, e.g., of the malignant or non-malignant type, e.g., cancer cells. Exemplary vitamins include vitamin A, E, and K. Other exemplary vitamins include are B

25   vitamin, e.g., folic acid, B12, riboflavin, biotin, pyridoxal or other vitamins or nutrients taken up by cancer cells. Also included are HSA and low density lipoprotein (LDL).

In another aspect, the ligand is a cell-permeation agent, preferably a helical cell-permeation agent. Preferably, the agent is amphipathic. An exemplary agent is a peptide such as tat or antennopedia. If the agent is a peptide, it can be modified, including a peptidylmimetic,

30   invertomers, non-peptide or pseudo-peptide linkages, and use of D-amino acids. The helical

agent is preferably an alpha-helical agent, which preferably has a lipophilic and a lipophobic phase.

The ligand can be a peptide or peptidomimetic. A peptidomimetic (also referred to herein as an oligopeptidomimetic) is a molecule capable of folding into a defined three-dimensional structure similar to a natural peptide. The attachment of peptide and peptidomimetics to iRNA agents can affect pharmacokinetic distribution of the iRNA, such as by enhancing cellular recognition and absorption. The peptide or peptidomimetic moiety can be about 5-50 amino acids long, *e.g.*, about 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids long (see Table 4, for example).

Table 4. Exemplary Cell Permeation Peptides

Cell Permeation Peptide	Amino acid Sequence	Reference
Penetratin	RQIKIWFQNRRMKWKK (SEQ ID NO:7)	Derossi *et al.*, J. Biol. Chem. 269:10444, 1994
Tat fragment (48-60)	GRKKRRQRRRPPQC (SEQ ID NO:8)	Vives *et al.*, J. Biol. Chem., 272:16010, 1997
Signal Sequence-based peptide	GALFLGWLGAAGSTMGAWSQPKKKRKV (SEQ ID NO:9)	Chaloin *et al.*, Biochem. Biophys. Res. Commun., 243:601, 1998
PVEC	LLIILRRRIRKQAHAHSK (SEQ ID NO:10)	Elmquist *et al.*, Exp. Cell Res., 269:237, 2001
Transportan	GWTLNSAGYLLKINLKALAALAKKIL (SEQ ID NO:11)	Pooga *et al.*, FASEB J., 12:67, 1998

Amphiphilic model peptide	KLALKLALKALKAALKLA (SEQ ID NO:12)	Oehlke *et al.*, Mol. Ther., 2:339, 2000
Arg9	RRRRRRRRR (SEQ ID NO:13)	Mitchell *et al.*, J. Pept. Res., 56:318, 2000
Bacterial cell wall permeating	KFFKFFKFFK (SEQ ID NO:14)	
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRN LVPRTES (SEQ ID NO:15)	
Cecropin P1	SWLSKTAKKLENSAKKRISEGIAIAIQGGP R (SEQ ID NO:16)	
α-defensin	ACYCRIPACIAGERRYGTCIYQGRLWAFC C (SEQ ID NO:17)	
b-defensin	DHYNCVSSGGQCLYSACPIFTKIQGTCYR GKAKCCK (SEQ ID NO:18)	
Bactenecin	RKCRIVVIRVCR (SEQ ID NO:19)	
PR-39	RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPP RFPPRFPGKR-NH2 (SEQ ID NO:20)	
Indolicidin	ILPWKWPWWPWRR-NH2 (SEQ ID NO:21)	

A peptide or peptidomimetic can be, for example, a cell permeation peptide, cationic peptide, amphipathic peptide, or hydrophobic peptide (e.g., consisting primarily of Tyr, Trp or Phe). The peptide moiety can be a dendrimer peptide, constrained peptide or crosslinked peptide. The peptide moiety can be an L-peptide or D-peptide. In another alternative, the peptide moiety can include a hydrophobic membrane translocation sequence (MTS). An exemplary hydrophobic MTS-containing peptide is RFGF having the amino acid sequence AAVALLPAVLLALLAP (SEQ ID NO:22). An RFGF analogue (e.g., amino acid sequence AALLPVLLAAP (SEQ ID NO:23)) containing a hydrophobic MTS can also be a targeting moiety. The peptide moiety can be a "delivery" peptide, which can carry large polar molecules including peptides, oligonucleotides, and protein across cell membranes. For example, sequences from the HIV Tat protein (GRKKRRQRRRPPQ (SEQ ID NO:24)) and the Drosophila Antennapedia protein (RQIKIWFQNRRMKWKK (SEQ ID NO:25)) have been found to be capable of functioning as delivery peptides. A peptide or peptidomimetic can be encoded by a random sequence of DNA, such as a peptide identified from a phage-display library, or one-bead-one-compound (OBOC) combinatorial library (Lam et al., Nature, 354:82-84, 1991). Preferably the peptide or peptidomimetic tethered to an iRNA agent via an incorporated monomer unit is a cell targeting peptide such as an arginine-glycine-aspartic acid (RGD)-peptide, or RGD mimic. A peptide moiety can range in length from about 5 amino acids to about 40 amino acids. The peptide moieties can have a structural modification, such as to increase stability or direct conformational properties. Any of the structural modifications described below can be utilized.

An RGD peptide moiety can be used to target a tumor cell, such as an endothelial tumor cell or a breast cancer tumor cell (Zitzmann et al., Cancer Res., 62:5139-43, 2002). An RGD peptide can facilitate targeting of an iRNA agent to tumors of a variety of other tissues, including the lung, kidney, spleen, or liver (Aoki et al., Cancer Gene Therapy 8:783-787, 2001). Preferably, the RGD peptide will facilitate targeting of an iRNA agent to the kidney. The RGD peptide can be linear or cyclic, and can be modified, e.g., glycosylated or methylated to facilitate targeting to specific tissues. For example, a glycosylated RGD peptide can deliver an iRNA agent to a tumor cell expressing $\alpha_v\beta_3$ (Haubner et al., Jour. Nucl. Med., 42:326-336, 2001).

Peptides that target markers enriched in proliferating cells can be used. E.g., RGD containing peptides and peptidomimetics can target cancer cells, in particular cells that exhibit an $\alpha_v\beta_3$ integrin. Thus, one could use RGD peptides, cyclic peptides containing RGD, RGD peptides that include D-amino acids, as well as synthetic RGD mimics. In addition to RGD, one

can use other moieties that target the α_v-β_3 integrin ligand. Generally, such ligands can be used to control proliferating cells and angiogeneis. Preferred conjugates of this type include an iRNA agent that targets PECAM-1, VEGF, or other cancer gene, e.g., a cancer gene described herein.

A "cell permeation peptide" is capable of permeating a cell, e.g., a microbial cell, such as a bacterial or fungal cell, or a mammalian cell, such as a human cell. A microbial cell-permeating peptide can be, for example, an α-helical linear peptide (e.g., LL-37 or Ceropin P1), a disulfide bond-containing peptide (e.g., α-defensin, β-defensin or bactenecin), or a peptide containing only one or two dominating amino acids (e.g., PR-39 or indolicidin). A cell permeation peptide can also include a nuclear localization signal (NLS). For example, a cell permeation peptide can be a bipartite amphipathic peptide, such as MPG, which is derived from the fusion peptide domain of HIV-1 gp41 and the NLS of SV40 large T antigen (Simeoni et al., Nucl. Acids Res. 31:2717-2724, 2003).

In one embodiment, a targeting peptide tethered to an RRMS can be an amphipathic α-helical peptide. Exemplary amphipathic α-helical peptides include, but are not limited to, cecropins, lycotoxins, paradaxins, buforin, CPF, bombinin-like peptide (BLP), cathelicidins, ceratotoxins, S. clava peptides, hagfish intestinal antimicrobial peptides (HFIAPs), magainines, brevinins-2, dermaseptins, melittins, pleurocidin, H_2A peptides, Xenopus peptides, esculentinis-1, and caerins. A number of factors will preferably be considered to maintain the integrity of helix stability. For example, a maximum number of helix stabilization residues will be utilized (e.g., leu, ala, or lys), and a minimum number helix destabilization residues will be utilized (e.g., proline, or cyclic monomeric units. The capping residue will be considered (for example Gly is an exemplary N-capping residue and/or C-terminal amidation can be used to provide an extra H-bond to stabilize the helix. Formation of salt bridges between residues with opposite charges, separated by i ± 3, or i ± 4 positions can provide stability. For example, cationic residues such as lysine, arginine, homo-arginine, ornithine or histidine can form salt bridges with the anionic residues glutamate or aspartate.

Peptide and petidomimetic ligands include those having naturally occurring or modified peptides, e.g., D or L peptides; α, β, or γ peptides; N-methyl peptides; azapeptides; peptides having one or more amide, i.e., peptide, linkages replaced with one or more urea, thiourea, carbamate, or sulfonyl urea linkages; or cyclic peptides.

Methods for making iRNA agents

iRNA agents can include modified or non-naturally occuring bases, e.g., bases described in copending and coowned United States Provisional Application Serial No. 60/463,772, filed on April 17, 2003, which is hereby incorporated by reference and/or in copending and coowned United States Provisional Application Serial No. 60/465,802, filed on April 25, 2003, which is hereby incorporated by reference. Monomers and iRNA agents which include such bases can be made by the methods found in United States Provisional Application Serial No. 60/463,772, filed on April 17, 2003, and/or in United States Provisional Application Serial No. 60/465,802, filed on April 25, 2003.

In addition, the invention includes iRNA agents having a modified or non-naturally occuring base and another element described herein. E.g., the invention includes an iRNA agent described herein, e.g., a palindromic iRNA agent, an iRNA agent having a non canonical pairing, an iRNA agent which targets a gene described herein, e.g., a gene active in the kidney, an iRNA agent having an architecture or structure described herein, an iRNA associated with an amphipathic delivery agent described herein, an iRNA associated with a drug delivery module described herein, an iRNA agent administered as described herein, or an iRNA agent formulated as described herein, which also incorporates a modified or non-naturally occuring base.

The synthesis and purification of oligonucleotide peptide conjugates can be performed by established methods. See, for example, Trufert *et al.*, Tetrahedron, 52:3005, 1996; and Manoharan, "Oligonucleotide Conjugates in Antisense Technology," in <u>Antisense Drug Technology</u>, ed. S.T. Crooke, Marcel Dekker, Inc., 2001.

In one embodiment of the invention, a peptidomimetic can be modified to create a constrained peptide that adopts a distinct and specific preferred conformation, which can increase the potency and selectivity of the peptide. For example, the constrained peptide can be an azapeptide (Gante, Synthesis, 405-413, 1989). An azapeptide is synthesized by replacing the α-carbon of an amino acid with a nitrogen atom without changing the structure of the amino acid side chain. For example, the azapeptide can be synthesized by using hydrazine in traditional peptide synthesis coupling methods, such as by reacting hydrazine with a "carbonyl donor," e.g., phenylchloroformate.

In one embodiment of the invention, a peptide or peptidomimetic (*e.g.*, a peptide or peptidomimetic tethered to an RRMS) can be an N-methyl peptide. N-methyl peptides are

composed of N-methyl amino acids, which provide an additional methyl group in the peptide backbone, thereby potentially providing additional means of resistance to proteolytic cleavage. N-methyl peptides can by synthesized by methods known in the art (see, for example, Lindgren *et al.*, Trends Pharmacol. Sci. 21:99, 2000; Cell Penetrating Peptides: Processes and Applications, Langel, ed., CRC Press, Boca Raton, FL, 2002; Fische *et al.*, Bioconjugate. Chem. 12: 825, 2001; Wander *et al.*, J. Am. Chem. Soc., 124:13382, 2002). For example, an Ant or Tat peptide can be an N-methyl peptide.

In one embodiment of the invention, a peptide or peptidomimetic (*e.g.*, a peptide or peptidomimetic tethered to an RRMS) can be a β-peptide. β-peptides form stable secondary structures such as helices, pleated sheets, turns and hairpins in solutions. Their cyclic derivatives can fold into nanotubes in the solid state. β-peptides are resistant to degradation by proteolytic enzymes. β-peptides can be synthesized by methods known in the art. For example, an Ant or Tat peptide can be a β-peptide.

In one embodiment of the invention, a peptide or peptidomimetic (*e.g.*, a peptide or peptidomimetic tethered to an RRMS) can be a oligocarbamate. Oligocarbamate peptides are internalized into a cell by a transport pathway facilitated by carbamate transporters. For example, an Ant or Tat peptide can be an oligocarbamate.

In one embodiment of the invention, a peptide or peptidomimetic (*e.g.*, a peptide or peptidomimetic tethered to an RRMS) can be an oligourea conjugate (or an oligothiourea conjugate), in which the amide bond of a peptidomimetic is replaced with a urea moiety. Replacement of the amide bond provides increased resistance to degradation by proteolytic enzymes, *e.g.*, proteolytic enzymes in the gastrointestinal tract. In one embodiment, an oligourea conjugate is tethered to an iRNA agent for use in oral delivery. The backbone in each repeating unit of an oligourea peptidomimetic can be extended by one carbon atom in comparison with the natural amino acid. The single carbon atom extension can increase peptide stability and lipophilicity, for example. An oligourea peptide can therefore be advantageous when an iRNA agent is directed for passage through a bacterial cell wall, or when an iRNA agent must traverse the blood-brain barrier, such as for the treatment of a neurological disorder. In one embodiment, a hydrogen bonding unit is conjugated to the oligourea peptide, such as to create an increased affinity with a receptor. For example, an Ant or Tat peptide can be an oligourea conjugate (or an oligothiourea conjugate).

The siRNA peptide conjugates of the invention can be affiliated with, *e.g.*, tethered to, RRMSs occurring at various positions on an iRNA agent. For example, a peptide can be terminally conjugated, on either the sense or the antisense strand, or a peptide can be bisconjugated (one peptide tethered to each end, one conjugated to the sense strand, and one conjugated to the antisense strand). In another option, the peptide can be internally conjugated, such as in the loop of a short hairpin iRNA agent. In yet another option, the peptide can be affiliated with a complex, such as a peptide-carrier complex.

A peptide-carrier complex consists of at least a carrier molecule, which can encapsulate one or more iRNA agents (such as for delivery to a biological system and/or a cell), and a peptide moiety tethered to the outside of the carrier molecule, such as for targeting the carrier complex to a particular tissue or cell type. A carrier complex can carry additional targeting molecules on the exterior of the complex, or fusogenic agents to aid in cell delivery. The one or more iRNA agents encapsulated within the carrier can be conjugated to lipophilic molecules, which can aid in the delivery of the agents to the interior of the carrier.

A carrier molecule or structure can be, for example, a micelle, a liposome (*e.g.*, a cationic liposome), a nanoparticle, a microsphere, or a biodegradable polymer. A peptide moiety can be tethered to the carrier molecule by a variety of linkages, such as a disulfide linkage, an acid labile linkage, a peptide-based linkage, an oxyamino linkage or a hydrazine linkage. For example, a peptide-based linkage can be a GFLG peptide. Certain linkages will have particular advantages, and the advantages (or disadvantages) can be considered depending on the tissue target or intended use. For example, peptide based linkages are stable in the blood stream but are susceptible to enzymatic cleavage in the lysosomes.

Targeting

The iRNA agents of the invention are particularly useful when targeted to the kidney. An iRNA agent can be targeted to the kidney by incorporation of an RRMS containing a ligand that targets the kidney.

A targeting agent that incorporates a sugar, *e.g.*, galactose and/or analogues thereof, can be useful. These agents target, for example, the parenchymal cells of the liver. For example, a targeting moiety can include more than one or preferably two or three galactose moieties, spaced about 15 angstroms from each other. The targeting moiety can alternatively be lactose (*e.g.*, three lactose moieties), which is glucose coupled to a galactose. The targeting moiety can also

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be N-Acetyl-Galactosamine, N-Ac-Glucosamine. A mannose or mannose-6-phosphate targeting moiety can be used for macrophage targeting.

Conjugation of an iRNA agent with a serum albumin (SA), such as human serum albumin, can also be used to target the iRNA agent to a non-kidney tissue, such as the liver.

An iRNA agent targeted to the kidney by an RRMS targeting moiety described herein can target a gene expressed in the kidney.

Ligands on RRMSs can include folic acid, glucose, cholesterol, cholic acid, Vitamin E, Vitamin K, or Vitamin A.

Definitions

The term "halo" refers to any radical of fluorine, chlorine, bromine or iodine.

The term "alkyl" refers to a hydrocarbon chain that may be a straight chain or branched chain, containing the indicated number of carbon atoms. For example, C_1-C_{12} alkyl indicates that the group may have from 1 to 12 (inclusive) carbon atoms in it. The term "haloalkyl" refers to an alkyl in which one or more hydrogen atoms are replaced by halo, and includes alkyl moieties in which all hydrogens have been replaced by halo (e.g., perfluoroalkyl). Alkyl and haloalkyl groups may be optionally inserted with O, N, or S. The terms "aralkyl" refers to an alkyl moiety in which an alkyl hydrogen atom is replaced by an aryl group. Aralkyl includes groups in which more than one hydrogen atom has been replaced by an aryl group. Examples of "aralkyl" include benzyl, 9-fluorenyl, benzhydryl, and trityl groups.

The term "alkenyl" refers to a straight or branched hydrocarbon chain containing 2-8 carbon atoms and characterized in having one or more double bonds. Examples of a typical alkenyl include, but not limited to, allyl, propenyl, 2-butenyl, 3-hexenyl and 3-octenyl groups. The term "alkynyl" refers to a straight or branched hydrocarbon chain containing 2-8 carbon atoms and characterized in having one or more triple bonds. Some examples of a typical alkynyl are ethynyl, 2-propynyl, and 3-methylbutynyl, and propargyl. The sp^2 and sp^3 carbons may optionally serve as the point of attachment of the alkenyl and alkynyl groups, respectively.

The term "alkoxy" refers to an -O-alkyl radical. The term "aminoalkyl" refers to an alkyl substituted with an aminoThe term "mercapto" refers to an -SH radical. The term "thioalkoxy" refers to an -S-alkyl radical.

The term "alkylene" refers to a divalent alkyl (*i.e.*, -R-), e.g., -CH$_2$-, -CH$_2$CH$_2$-, and -CH$_2$CH$_2$CH$_2$-.   The term "alkylenedioxo" refers to a divalent species of the structure -O-R-O-, in which R represents an alkylene.

The term "aryl" refers to an aromatic monocyclic, bicyclic, or tricyclic hydrocarbon ring system, wherein any ring atom capable of substitution can be substituted by a substituent. Examples of aryl moieties include, but are not limited to, phenyl, naphthyl, and anthracenyl.

The term "cycloalkyl" as employed herein includes saturated cyclic, bicyclic, tricyclic,or polycyclic hydrocarbon groups having 3 to 12 carbons, wherein any ring atom capable of substitution can be substituted by a substituent. The cycloalkyl groups herein described may also contain fused rings. Fused rings are rings that share a common carbon-carbon bond. Examples of cycloalkyl moieties include, but are not limited to, cyclohexyl, adamantyl, and norbornyl.

The term "heterocyclyl" refers to a nonaromatic 3-10 membered monocyclic, 8-12 membered bicyclic, or 11-14 membered tricyclic ring system having 1-3 heteroatoms if monocyclic, 1-6 heteroatoms if bicyclic, or 1-9 heteroatoms if tricyclic, said heteroatoms selected from O, N, or S (e.g., carbon atoms and 1-3, 1-6, or 1-9 heteroatoms of N, O, or S if monocyclic, bicyclic, or tricyclic, respectively), wherein any ring atom capable of substitution can be substituted by a substituent. The heterocyclyl groups herein described may also contain fused rings. Fused rings are rings that share a common carbon-carbon bond. Examples of heterocyclyl include, but are not limited to tetrahydrofuranyl, tetrahydropyranyl, piperidinyl, morpholino, pyrrolinyl and pyrrolidinyl.

The term "heteroaryl" refers to an aromatic 5-8 membered monocyclic, 8-12 membered bicyclic, or 11-14 membered tricyclic ring system having 1-3 heteroatoms if monocyclic, 1-6 heteroatoms if bicyclic, or 1-9 heteroatoms if tricyclic, said heteroatoms selected from O, N, or S (e.g., carbon atoms and 1-3, 1-6, or 1-9 heteroatoms of N, O, or S if monocyclic, bicyclic, or tricyclic, respectively), wherein any ring atom capable of substitution can be substituted by a substituent.

The term "oxo" refers to an oxygen atom, which forms a carbonyl when attached to carbon, an N-oxide when attached to nitrogen, and a sulfoxide or sulfone when attached to sulfur.

The term "acyl" refers to an alkylcarbonyl, cycloalkylcarbonyl, arylcarbonyl, heterocyclylcarbonyl, or heteroarylcarbonyl substituent, any of which may be further substituted by substituents.

The term "substituents" refers to a group "substituted" on an alkyl, cycloalkyl, alkenyl, alkynyl, heterocyclyl, heterocycloalkenyl, cycloalkenyl, aryl, or heteroaryl group at any atom of that group. Suitable substituents include, without limitation, alkyl, alkenyl, alkynyl, alkoxy, halo, hydroxy, cyano, nitro, amino, SO_3H, sulfate, phosphate, perfluoroalkyl, perfluoroalkoxy, methylenedioxy, ethylenedioxy, carboxyl, oxo, thioxo, imino (alkyl, aryl, aralkyl), $S(O)_n$alkyl (where n is 0-2), $S(O)_n$ aryl (where n is 0-2), $S(O)_n$ heteroaryl (where n is 0-2), $S(O)_n$ heterocyclyl (where n is 0-2), amine (mono-, di-, alkyl, cycloalkyl, aralkyl, heteroaralkyl, and combinations thereof), ester (alkyl, aralkyl, heteroaralkyl), amide (mono-, di-, alkyl, aralkyl, heteroaralkyl, and combinations thereof), sulfonamide (mono-, di-, alkyl, aralkyl, heteroaralkyl, and combinations thereof), unsubstituted aryl, unsubstituted heteroaryl, unsubstituted heterocyclyl, and unsubstituted cycloalkyl. In one aspect, the substituents on a group are independently any one single, or any subset of the aforementioned substituents.

The terms "adeninyl, cytosinyl, guaninyl, thyminyl, and uracilyl" and the like refer to radicals of adenine, cytosine, guanine, thymine, and uracil.

As used herein, an "unusual" nucleobase can include any one of the following:

2-methyladeninyl,

N6-methyladeninyl,

2-methylthio-N6-methyladeninyl,

N6-isopentenyladeninyl,

2-methylthio-N6-isopentenyladeninyl,

N6-(cis-hydroxyisopentenyl)adeninyl,

2-methylthio-N6-(cis-hydroxyisopentenyl) adeninyl,

N6-glycinylcarbamoyladeninyl,

N6-threonylcarbamoyladeninyl,

2-methylthio-N6-threonyl carbamoyladeninyl,

N6-methyl-N6-threonylcarbamoyladeninyl,

N6-hydroxynorvalylcarbamoyladeninyl,

2-methylthio-N6-hydroxynorvalyl carbamoyladeninyl,

N6,N6-dimethyladeninyl,

3-methylcytosinyl,

5-methylcytosinyl,

2-thiocytosinyl,

5-formylcytosinyl,

,
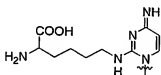
N4-methylcytosinyl,

5-hydroxymethylcytosinyl,

1-methylguaninyl,

N2-methylguaninyl,

7-methylguaninyl,

N2,N2-dimethylguaninyl,

beta-galactosyl

beta-mannosyl

N2,7-dimethylguaninyl,

5
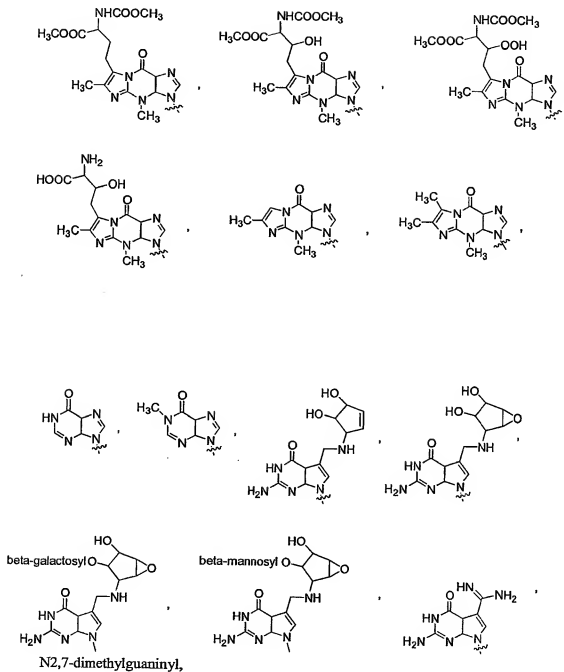
N2,N2,7-trimethylguaninyl,

1-methylguaninyl,

7-cyano-7-deazaguaninyl,

7-aminomethyl-7-deazaguaninyl,

pseudouracilyl,

dihydrouracilyl,

5-methyluracilyl,

1-methylpseudouracilyl,

2-thiouracilyl,

4-thiouracilyl,

2-thiothyminyl

5-methyl-2-thiouracilyl,

3-(3-amino-3-carboxypropyl)uracilyl,

5-hydroxyuracilyl,

5-methoxyuracilyl,

uracilyl 5-oxyacetic acid,

uracilyl 5-oxyacetic acid methyl ester,

5-(carboxyhydroxymethyl)uracilyl,

5-(carboxyhydroxymethyl)uracilyl methyl ester,

5-methoxycarbonylmethyluracilyl,

5-methoxycarbonylmethyl-2-thiouracilyl,

5-aminomethyl-2-thiouracilyl,

5-methylaminomethyluracilyl,

5-methylaminomethyl-2-thiouracilyl,

5-methylaminomethyl-2-selenouracilyl,

5-carbamoylmethyluracilyl,

5-carboxymethylaminomethyluracilyl,

5-carboxymethylaminomethyl-2-thiouracilyl,

3-methyluracilyl,

1-methyl-3-(3-amino-3-carboxypropyl) pseudouracilyl,

5-carboxymethyluracilyl,

5-methyldihydrouracilyl, or

3-methylpseudouracilyl.

Palindromes

An RNA, e.g., an iRNA agent, can have a palindrome structure as described herein and those described in one or more of United States Provisional Application Serial No. 60/452,682, filed March 7, 2003; United States Provisional Application Serial No. 60/462,894, filed April 14, 2003; and International Application No. PCT/US04/07070, filed March 8, 2004, all of which are hereby incorporated by reference. The iRNA agents of the invention can target more than one RNA region. For example, an iRNA agent can include a first and second sequence that are sufficiently complementary to each other to hybridize. The first sequence can be complementary to a first target RNA region and the second sequence can be complementary to a second target RNA region. The first and second sequences of the iRNA agent can be on different RNA strands, and the mismatch between the first and second sequences can be less than 50%, 40%, 30%, 20%, 10%, 5%, or 1%. The first and second sequences of the iRNA agent are on the same RNA strand, and in a related embodiment more than 50%, 60%, 70%, 80%, 90%, 95%, or 1% of the iRNA agent can be in bimolecular form. The first and second sequences of the iRNA agent can be fully complementary to each other.

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The first target RNA region can be encoded by a first gene and the second target RNA region can encoded by a second gene, or the first and second target RNA regions can be different regions of an RNA from a single gene. The first and second sequences can differ by at least 1 nucleotide.

The first and second target RNA regions can be on transcripts encoded by first and second sequence variants, e.g., first and second alleles, of a gene. The sequence variants can be mutations, or polymorphisms, for example. The first target RNA region can include a nucleotide substitution, insertion, or deletion relative to the second target RNA region, or the second target RNA region can a mutant or variant of the first target region.

The first and second target RNA regions can comprise viral or human RNA regions. The first and second target RNA regions can also be on variant transcripts of an oncogene or include different mutations of a tumor suppressor gene transcript. In addition, the first and second target RNA regions can correspond to hot-spots for genetic variation.

The compositions of the invention can include mixtures of iRNA agent molecules. For example, one iRNA agent can contain a first sequence and a second sequence sufficiently complementary to each other to hybridize, and in addition the first sequence is complementary to a first target RNA region and the second sequence is complementary to a second target RNA region. The mixture can also include at least one additional iRNA agent variety that includes a third sequence and a fourth sequence sufficiently complementary to each other to hybridize, and where the third sequence is complementary to a third target RNA region and the fourth sequence is complementary to a fourth target RNA region. In addition, the first or second sequence can be sufficiently complementary to the third or fourth sequence to be capable of hybridizing to each other. The first and second sequences can be on the same or different RNA strands, and the third and fourth sequences can be on the same or different RNA strands.

The target RNA regions can be variant sequences of a viral or human RNA, and in certain embodiments, at least two of the target RNA regions can be on variant transcripts of an oncogene or tumor suppressor gene. The target RNA regions can correspond to genetic hot-spots.

Methods of making an iRNA agent composition can include obtaining or providing information about a region of an RNA of a target gene (e.g., a viral or human gene, or an oncogene or tumor suppressor, e.g., p53), where the region has high variability or mutational

frequency (e.g., in humans). In addition, information about a plurality of RNA targets within the region can be obtained or provided, where each RNA target corresponds to a different variant or mutant of the gene (e.g., a region including the codon encoding p53 248Q and/or p53 249S). The iRNA agent can be constructed such that a first sequence is complementary to a first of the plurality of variant RNA targets (e.g., encoding 249Q) and a second sequence is complementary to a second of the plurality of variant RNA targets (e.g., encoding 249S), and the first and second sequences can be sufficiently complementary to hybridize.

Sequence analysis, e.g., to identify common mutants in the target gene, can be used to identify a region of the target gene that has high variability or mutational frequency. A region of the target gene having high variability or mutational frequency can be identified by obtaining or providing genotype information about the target gene from a population.

Expression of a target gene can be modulated, e.g., downregulated or silenced, by providing an iRNA agent that has a first sequence and a second sequence sufficiently complementary to each other to hybridize. In addition, the first sequence can be complementary to a first target RNA region and the second sequence can be complementary to a second target RNA region.

An iRNA agent can include a first sequence complementary to a first variant RNA target region and a second sequence complementary to a second variant RNA target region. The first and second variant RNA target regions can correspond to first and second variants or mutants of a target gene, e.g., viral gene, tumor suppressor or oncogene. The first and second variant target RNA regions can include allelic variants, mutations (e.g., point mutations), or polymorphisms of the target gene. The first and second variant RNA target regions can correspond to genetic hot-spots.

A plurality of iRNA agents (e.g., a panel or bank) can be provided.

Other than Canonical Watson-Crick Duplex Structures

An RNA, e.g., an iRNA agent can include monomers which can form other than a canonical Watson-Crick pairing with another monomer, e.g., a monomer on another strand, such as those described herein and those described in United States Provisional Application Serial No. 60/465,665, filed April 25, 2003, and International Application No. PCT/US04/07070, filed March 8, 2004, both of which are hereby incorporated by reference.

The use of "other than canonical Watson-Crick pairing" between monomers of a duplex can be used to control, often to promote, melting of all or part of a duplex. The iRNA agent can include a monomer at a selected or constrained position that results in a first level of stability in the iRNA agent duplex (e.g., between the two separate molecules of a double stranded iRNA agent) and a second level of stability in a duplex between a sequence of an iRNA agent and another sequence molecule, e.g., a target or off-target sequence in a subject. In some cases the second duplex has a relatively greater level of stability, e.g., in a duplex between an anti-sense sequence of an iRNA agent and a target mRNA. In this case one or more of the monomers, the position of the monomers in the iRNA agent, and the target sequence (sometimes referred to herein as the selection or constraint parameters), are selected such that the iRNA agent duplex is has a comparatively lower free energy of association (which while not wishing to be bound by mechanism or theory, is believed to contribute to efficacy by promoting disassociation of the duplex iRNA agent in the context of the RISC) while the duplex formed between an anti-sense targeting sequence and its target sequence, has a relatively higher free energy of association (which while not wishing to be bound by mechanism or theory, is believed to contribute to efficacy by promoting association of the anti-sense sequence and the target RNA).

In other cases the second duplex has a relatively lower level of stability, e.g., in a duplex between a sense sequence of an iRNA agent and an off-target mRNA. In this case one or more of the monomers, the position of the monomers in the iRNA agent, and an off-target sequence, are selected such that the iRNA agent duplex is has a comparatively higher free energy of association while the duplex formed between a sense targeting sequence and its off-target sequence, has a relatively lower free energy of association (which while not wishing to be bound by mechanism or theory, is believed to reduce the level of off-target silencing by contribute to efficacy by promoting disassociation of the duplex formed by the sense strand and the off-target sequence).

Thus, inherent in the structure of the iRNA agent is the property of having a first stability for the intra-iRNA agent duplex and a second stability for a duplex formed between a sequence from the iRNA agent and another RNA, e.g., a target mRNA. As discussed above, this can be accomplished by judicious selection of one or more of the monomers at a selected or constrained position, the selection of the position in the duplex to place the selected or constrained position, and selection of the sequence of a target sequence (e.g., the particular region of a target gene which is to be targeted). The iRNA agent sequences which satisfy these requirements are sometimes referred herein as constrained sequences. Exercise of the constraint or selection

parameters can e, e.g., by inspection, or by computer assisted methods. Exercise of the parameters can result in selection of a target sequence and of particular monomers to give a desired result in terms of the stability, or relative stability, of a duplex.

Thus, in another aspect, the invention features, an iRNA agent which includes: a first sequence which targets a first target region and a second sequence which targets a second target region. The first and second sequences have sufficient complementarity to each other to hybridize, e.g., under physiological conditions, e.g., under physiological conditions but not in contact with a helicase or other unwinding enzyme. In a duplex region of the iRNA agent, at a selected or constrained position, the first target region has a first monomer, and the second target region has a second monomer. The first and second monomers occupy complementary or corresponding positions. One, and preferably both monomers are selected such that the stability of the pairing of the monomers contribute to a duplex between the first and second sequence will differ form the stability of the pairing between the first or second sequence with a target sequence.

Usually, the monomers will be selected (selection of the target sequence may be required as well) such that they form a pairing in the iRNA agent duplex which has a lower free energy of dissociation, and a lower Tm, than will be possessed by the paring of the monomer with its complementary monomer in a duplex between the iRNA agent sequence and a target RNA duplex.

The constraint placed upon the monomers can be applied at a selected site or at more than one selected site. By way of example, the constraint can be applied at more than 1, but less than 3, 4, 5, 6, or 7 sites in an iRNA agent duplex.

A constrained or selected site can be present at a number of positions in the iRNA agent duplex. E.g., a constrained or selected site can be present within 3, 4, 5, or 6 positions from either end, 3' or 5' of a duplexed sequence. A constrained or selected site can be present in the middle of the duplex region, e.g., it can be more than 3, 4, 5, or 6, positions from the end of a duplexed region.

In some embodiment the duplex region of the iRNA agent will have, mismatches, in addition to the selected or constrained site or sites. Preferably it will have no more than 1, 2, 3, 4, or 5 bases, which do not form canonical Watson-Crick pairs or which do not hybridize. Overhangs are discussed in detail elsewhere herein but are preferably about 2 nucleotides in

length. The overhangs can be complementary to the gene sequences being targeted or can be other sequence. TT is a preferred overhang sequence. The first and second iRNA agent sequences can also be joined, e.g., by additional bases to form a hairpin, or by other non-base linkers.

The monomers can be selected such that: first and second monomers are naturally occurring ribonuceotides, or modified ribonucleotides having naturally occurring bases, and when occupying complemetary sites either do not pair and have no substantial level of H-bonding, or form a non canonical Watson-Crick pairing and form a non-canonical pattern of H bonding, which usually have a lower free energy of dissociation than seen in a canonical Watson-Crick pairing, or otherwise pair to give a free energy of association which is less than that of a preselected value or is less, e.g., than that of a canonical pairing. When one (or both) of the iRNA agent sequences duplexes with a target, the first (or second) monomer forms a canonical Watson-Crick pairing with the base in the complemetary position on the target, or forms a non canonical Watson-Crick pairing having a higher free energy of dissociation and a higher Tm than seen in the paring in the iRNA agent. The classical Watson-Crick parings are as follows: A-T, G-C, and A-U. Non-canonical Watson-Crick pairings are known in the art and can include, U-U, G-G, G-A$_{trans}$, G-A$_{cis}$, and GU.

The monomer in one or both of the sequences is selected such that, it does not pair, or forms a pair with its corresponding monomer in the other sequence which minimizes stability (e.g., the H bonding formed between the monomer at the selected site in the one sequence and its monomer at the corresponding site in the other sequence are less stable than the H bonds formed by the monomer one (or both) of the sequences with the respective target sequence. The monomer is one or both strands is also chosen to promote stability in one or both of the duplexes made by a strand and its target sequence. E.g., one or more of the monomers and the target sequences are selected such that at the selected or constrained position, there is are no H bonds formed, or a non canonical pairing is formed in the iRNA agent duplex, or otherwise they otherwise pair to give a free energy of association which is less than that of a preselected value or is less, e.g., than that of a canonical pairing, but when one (or both) sequences form a duplex with the respective target, the pairing at the selected or constrained site is a canonical Watson-Crick paring.

The inclusion of such a monomers will have one or more of the following effects: it will destabilize the iRNA agent duplex, it will destabilize interactions between the sense sequence

and unintended target sequences, sometimes referred to as off-target sequences, and duplex interactions between the a sequence and the intended target will not be destabilized.

By way of example:

The monomer at the selected site in the first sequence includes an A (or a modified base which pairs with T), and the monomer in at the selected position in the second sequence is chosen from a monomer which will not pair or which will form a non-canonical pairing, e.g., G. These will be useful in applications wherein the target sequence for the first sequence has a T at the selected position. In embodiments where both target duplexes are stabilized it is useful wherein the target sequence for the second strand has a monomer which will form a canonical Watson-Crick pairing with the monomer selected for the selected position in the second strand.

The monomer at the selected site in the first sequence includes U (or a modified base which pairs with A), and the monomer in at the selected position in the second sequence is chosen from a monomer which will not pair or which will form a non-canonical pairing, e.g., U or G. These will be useful in applications wherein the target sequence for the first sequence has a T at the selected position. In embodiments where both target duplexes are stabilized it is useful wherein the target sequence for the second strand has a monomer which will form a canonical Watson-Crick pairing with the monomer selected for the selected position in the second strand.

The monomer at the selected site in the first sequence includes a G (or a modified base which pairs with C), and the monomer in at the selected position in the second sequence is chosen from a monomer which will not pair or which will form a non-canonical pairing, e.g., G, A_{cis}, A_{trans}, or U. These will be useful in applications wherein the target sequence for the first sequence has a T at the selected position. In embodiments where both target duplexes are stabilized it is useful wherein the target sequence for the second strand has a monomer which will form a canonical Watson-Crick pairing with the monomer selected for the selected position in the second strand.

The monomer at the selected site in the first sequence includes a C (or a modified base which pairs with G), and the monomer in at the selected position in the second sequence is chosen a monomer which will not pair or which will form a non-canonical pairing. These will be useful in applications wherein the target sequence for the first sequence has a T at the selected position. In embodiments where both target duplexes are stabilized it is useful wherein the target

sequence for the second strand has a monomer which will form a canonical Watson-Crick pairing with the monomer selected for the selected position in the second strand.

A non-naturally occurring or modified monomer or monomers can be chosen such that when a non-naturally occurring or modified monomer occupies a positions at the selected or constrained position in an iRNA agent they exhibit a first free energy of dissociation and when one (or both) of them pairs with a naturally occurring monomer, the pair exhibits a second free energy of dissociation, which is usually higher than that of the pairing of the first and second monomers. E.g., when the first and second monomers occupy complementary positions they either do not pair and have no substantial level of H-bonding, or form a weaker bond than one of them would form with a naturally occurring monomer, and reduce the stability of that duplex, but when the duplex dissociates at least one of the strands will form a duplex with a target in which the selected monomer will promote stability, e.g., the monomer will form a more stable pair with a naturally occurring monomer in the target sequence than the pairing it formed in the iRNA agent.

An example of such a pairing is 2-amino A and either of a 2-thio pyrimidine analog of U or T.

When placed in complementary positions of the iRNA agent these monomers will pair very poorly and will minimize stability. However, a duplex is formed between 2 amino A and the U of a naturally occurring target, or a duplex is between 2-thio U and the A of a naturally occurring target or 2-thio T and the A of a naturally occurring target will have a relatively higher free energy of dissociation and be more stable. This is shown in the FIG. 1.

The pair shown in FIG. 1 (the 2-amino A and the 2-s U and T) is exemplary. In another embodiment, the monomer at the selected position in the sense strand can be a universal pairing moiety. A universal pairing agent will form some level of H bonding with more than one and preferably all other naturally occurring monomers. An examples of a universal pairing moiety is a monomer which includes 3-nitro pyrrole. (Examples of other candidate universal base analogs can be found in the art, e.g., in Loakes, 2001, NAR 29: 2437-2447, hereby incorporated by reference. Examples can also be found in the section on Universal Bases below.) In these cases the monomer at the corresponding position of the anti-sense strand can be chosen for its ability to form a duplex with the target and can include, e.g., A, U, G, or C.

iRNA agents of the invention can include:

A sense sequence, which preferably does not target a sequence in a subject, and an anti-sense sequence, which targets a target gene in a subject. The sense and anti-sense sequences have sufficient complementarity to each other to hybridize hybridize, e.g., under physiological conditions, e.g., under physiological conditions but not in contact with a helicase or other unwinding enzyme. In a duplex region of the iRNA agent, at a selected or constrained position, the monomers are selected such that:

The monomer in the sense sequence is selected such that, it does not pair, or forms a pair with its corresponding monomer in the anti-sense strand which minimizes stability (e.g., the H bonding formed between the monomer at the selected site in the sense strand and its monomer at the corresponding site in the anti-sense strand are less stable than the H bonds formed by the monomer of the anti-sense sequence and its canonical Watson-Crick partner or, if the monomer in the anti-sense strand includes a modified base, the natural analog of the modified base and its canonical Watson-Crick partner);

The monomer is in the corresponding position in the anti-sense strand is selected such that it maximizes the stability of a duplex it forms with the target sequence, e.g., it forms a canonical Watson-Crick paring with the monomer in the corresponding position on the target stand;

Optionally, the monomer in the sense sequence is selected such that, it does not pair, or forms a pair with its corresponding monomer in the anti-sense strand which minimizes stability with an off-target sequence.

The inclusion of such a monomers will have one or more of the following effects: it will destabilize the iRNA agent duplex, it will destabilize interactions between the sense sequence and unintended target sequences, sometimes referred to as off-target sequences, and duplex interactions between the anti-sense strand and the intended target will not be destabilized.

The constraint placed upon the monomers can be applied at a selected site or at more than one selected site. By way of example, the constraint can be applied at more than 1, but less than 3, 4, 5, 6, or 7 sites in an iRNA agent duplex.

A constrained or selected site can be present at a number of positions in the iRNA agent duplex. E.g., a constrained or selected site can be present within 3, 4, 5, or 6 positions from

either end, 3' or 5' of a duplexed sequence. A constrained or selected site can be present in the middle of the duplex region, e.g., it can be more than 3, 4, 5, or 6, positions from the end of a duplexed region.

In some embodiment the duplex region of the iRNA agent will have, mismatches, in addition to the selected or constrained site or sites. Preferably it will have no more than 1, 2, 3, 4, or 5 bases, which do not form canonical Watson-Crick pairs or which do not hybridize. Overhangs are discussed in detail elsewhere herein but are preferably about 2 nucleotides in length. The overhangs can be complementary to the gene sequences being targeted or can be other sequence. TT is a preferred overhang sequence. The first and second iRNA agent sequences can also be joined, e.g., by additional bases to form a hairpin, or by other non-base linkers.

The monomers can be selected such that: first and second monomers are naturally occurring ribonuceotides, or modified ribonucleotides having naturally occurring bases, and when occupying complemetary sites either do not pair and have no substantial level of H-bonding, or form a non canonical Watson-Crick pairing and form a non-canonical pattern of H bonding, which usually have a lower free energy of dissociation than seen in a canonical Watson-Crick pairing, or otherwise pair to give a free energy of association which is less than that of a preselected value or is less, e.g., than that of a canonical pairing. When one (or both) of the iRNA agent sequences duplexes with a target, the first (or second) monomer forms a canonical Watson-Crick pairing with the base in the complemetary position on the target, or forms a non canonical Watson-Crick pairing having a higher free energy of dissociation and a higher Tm than seen in the paring in the iRNA agent. The classical Watson-Crick parings are as follows: A-T, G-C, and A-U. Non-canonical Watson-Crick pairings are known in the art and can include, U-U, G-G, G-A$_{trans}$, G-A$_{cis}$, and GU.

The monomer in one or both of the sequences is selected such that, it does not pair, or forms a pair with its corresponding monomer in the other sequence which minimizes stability (e.g., the H bonding formed between the monomer at the selected site in the one sequence and its monomer at the corresponding site in the other sequence are less stable than the H bonds formed by the monomer one (or both) of the sequences with the respective target sequence. The monomer is one or both strands is also chosen to promote stability in one or both of the duplexes made by a strand and its target sequence. E.g., one or more of the monomers and the target sequences are selected such that at the selected or constrained position, there is are no H bonds

formed, or a non canonical pairing is formed in the iRNA agent duplex, or otherwise they otherwise pair to give a free energy of association which is less than that of a preselected value or is less, e.g., than that of a canonical pairing, but when one (or both) sequences form a duplex with the respective target, the pairing at the selected or constrained site is a canonical Watson-5   Crick paring.

The inclusion of such a monomers will have one or more of the following effects: it will destabilize the iRNA agent duplex, it will destabilize interactions between the sense sequence and unintended target sequences, sometimes referred to as off-target sequences, and duplex interactions between the a sequence and the intended target will not be destabilized.

10   By way of example:

The monomer at the selected site in the first sequence includes an A (or a modified base which pairs with T), and the monomer in at the selected position in the second sequence is chosen from a monomer which will not pair or which will form a non-canonical pairing, e.g., G. These will be useful in applications wherein the target sequence for the first sequence has a T at 15   the selected position. In embodiments where both target duplexes are stabilized it is useful wherein the target sequence for the second strand has a monomer which will form a canonical Watson-Crick pairing with the monomer selected for the selected position in the second strand.

The monomer at the selected site in the first sequence includes U (or a modified base which pairs with A), and the monomer in at the selected position in the second sequence is 20   chosen from a monomer which will not pair or which will form a non-canonical pairing; e.g., U or G. These will be useful in applications wherein the target sequence for the first sequence has a T at the selected position. In embodiments where both target duplexes are stabilized it is useful wherein the target sequence for the second strand has a monomer which will form a canonical Watson-Crick pairing with the monomer selected for the selected position in the second strand.

25   The monomer at the selected site in the first sequence includes a G (or a modified base which pairs with C), and the monomer in at the selected position in the second sequence is chosen from a monomer which will not pair or which will form a non-canonical pairing, e.g., G, A_{cis}, A_{trans}, or U. These will be useful in applications wherein the target sequence for the first sequence has a T at the selected position. In embodiments where both target duplexes are 30   stabilized it is useful wherein the target sequence for the second strand has a monomer which

will form a canonical Watson-Crick pairing with the monomer selected for the selected position
in the second strand.

The monomer at the selected site in the first sequence includes a C (or a modified base
which pairs with G), and the monomer in at the selected position in the second sequence is
chosen a monomer which will not pair or which will form a non-canonical pairing. These will be
useful in applications wherein the target sequence for the first sequence has a T at the selected
position. In embodiments where both target duplexes are stabilized it is useful wherein the target
sequence for the second strand has a monomer which will form a canonical Watson-Crick
pairing with the monomer selected for the selected position in the second strand.

A non-naturally occurring or modified monomer or monomers can be chosen such that
when a non-naturally occurring or modified monomer occupies a positions at the selected or
constrained position in an iRNA agent they exhibit a first free energy of dissociation and when
one (or both) of them pairs with a naturally occurring monomer, the pair exhibits a second free
energy of dissociation, which is usually higher than that of the pairing of the first and second
monomers. E.g., when the first and second monomers occupy complementary positions they
either do not pair and have no substantial level of H-bonding, or form a weaker bond than one of
them would form with a naturally occurring monomer, and reduce the stability of that duplex, but
when the duplex dissociates at least one of the strands will form a duplex with a target in which
the selected monomer will promote stability, e.g., the monomer will form a more stable pair with
a naturally occurring monomer in the target sequence than the pairing it formed in the iRNA
agent.

An example of such a pairing is 2-amino A and either of a 2-thio pyrimidine analog of U
or T.

When placed in complementary positions of the iRNA agent these monomers will pair
very poorly and will minimize stability. However, a duplex is formed between 2 amino A and
the U of a naturally occurring target, or a duplex is between 2-thio U and the A of a naturally
occurring target or 2-thio T and the A of a naturally occurring target will have a relatively higher
free energy of dissociation and be more stable.

The monomer at the selected position in the sense strand can be a universal pairing
moiety. A universal pairing agent will form some level of H bonding with more than one and
preferably all other naturally occurring monomers. An examples of a universal pairing moiety is

a monomer which includes 3-nitro pyrrole. (Examples of other candidate universal base analogs can be found in the art, e.g., in Loakes, 2001, NAR 29: 2437-2447, hereby incorporated by reference. Examples can also be found in the section on Universal Bases below.) In these cases the monomer at the corresponding position of the anti-sense strand can be chosen for its ability to form a duplex with the target and can include, e.g., A, U, G, or C.

iRNA agents of the invention can include:

A sense sequence, which preferably does not target a sequence in a subject, and an anti-sense sequence, which targets a target gene in a subject. The sense and anti-sense sequences have sufficient complementarity to each other to hybridize hybridize, e.g., under physiological conditions, e.g., under physiological conditions but not in contact with a helicase or other unwinding enzyme. In a duplex region of the iRNA agent, at a selected or constrained position, the monomers are selected such that:

The monomer in the sense sequence is selected such that, it does not pair, or forms a pair with its corresponding monomer in the anti-sense strand which minimizes stability (e.g., the H bonding formed between the monomer at the selected site in the sense strand and its monomer at the corresponding site in the anti-sense strand are less stable than the H bonds formed by the monomer of the anti-sense sequence and its canonical Watson-Crick partner or, if the monomer in the anti-sense strand includes a modified base, the natural analog of the modified base and its canonical Watson-Crick partner);

The monomer is in the corresponding position in the anti-sense strand is selected such that it maximizes the stability of a duplex it forms with the target sequence, e.g., it forms a canonical Watson-Crick paring with the monomer in the corresponding position on the target stand;

Optionally, the monomer in the sense sequence is selected such that, it does not pair, or forms a pair with its corresponding monomer in the anti-sense strand which minimizes stability with an off-target sequence.

The inclusion of such a monomers will have one or more of the following effects: it will destabilize the iRNA agent duplex, it will destabilize interactions between the sense sequence and unintended target sequences, sometimes referred to as off-target sequences, and duplex interactions between the anti-sense strand and the intended target will not be destabilized.

The constraint placed upon the monomers can be applied at a selected site or at more than one selected site. By way of example, the constraint can be applied at more than 1, but less than 3, 4, 5, 6, or 7 sites in an iRNA agent duplex.

A constrained or selected site can be present at a number of positions in the iRNA agent duplex. E.g., a constrained or selected site can be present within 3, 4, 5, or 6 positions from either end, 3' or 5' of a duplexed sequence. A constrained or selected site can be present in the middle of the duplex region, e.g., it can be more than 3, 4, 5, or 6, positions from the end of a duplexed region.

The iRNA agent can be selected to target a broad spectrum of genes, including any of the genes described herein.

In a preferred embodiment the iRNA agent has an architecture (architecture refers to one or more of overall length, length of a duplex region, the presence, number, location, or length of overhangs, sing strand versus double strand form) described herein.

E.g., the iRNA agent can be less than 30 nucleotides in length, e.g., 21-23 nucleotides. Preferably, the iRNA is 21 nucleotides in length and there is a duplex region of about 19 pairs. In one embodiment, the iRNA is 21 nucleotides in length, and the duplex region of the iRNA is 19 nucleotides. In another embodiment, the iRNA is greater than 30 nucleotides in length.

In some embodiment the duplex region of the iRNA agent will have, mismatches, in addition to the selected or constrained site or sites. Preferably it will have no more than 1, 2, 3, 4, or 5 bases, which do not form canonical Watson-Crick pairs or which do not hybridize. Overhangs are discussed in detail elsewhere herein but are preferably about 2 nucleotides in length. The overhangs can be complementary to the gene sequences being targeted or can be other sequence. TT is a preferred overhang sequence. The first and second iRNA agent sequences can also be joined, e.g., by additional bases to form a hairpin, or by other non-base linkers.

One or more selection or constraint parameters can be exercised such that: monomers at the selected site in the sense and anti-sense sequences are both naturally occurring ribonucleotides, or modified ribonucleotides having naturally occurring bases, and when occupying complementary sites in the iRNA agent duplex either do not pair and have no substantial level of H-bonding, or form a non-canonical Watson-Crick pairing and thus form a non-canonical pattern of H bonding, which generally have a lower free energy of dissociation

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than seen in a Watson-Crick pairing, or otherwise pair to give a free energy of association which is less than that of a preselected value or is less, e.g., than that of a canonical pairing. When one, usually the anti-sense sequence of the iRNA agent sequences forms a duplex with another sequence, generally a sequence in the subject, and generally a target sequence, the monomer forms a classic Watson-Crick pairing with the base in the complementary position on the target, or forms a non-canonical Watson-Crick pairing having a higher free energy of dissociation and a higher Tm than seen in the paring in the iRNA agent. Optionally, when the other sequence of the iRNA agent, usually the sense sequences forms a duplex with another sequence, generally a sequence in the subject, and generally an off-target sequence, the monomer fails to forms a canonical Watson-Crick pairing with the base in the complementary position on the off target sequence, e.g., it forms or forms a non-canonical Watson-Crick pairing having a lower free energy of dissociation and a lower Tm.

By way of example:

the monomer at the selected site in the anti-sense stand includes an A (or a modified base which pairs with T), the corresponding monomer in the target is a T, and the sense strand is chosen from a base which will not pair or which will form a noncanonical pair, e.g., G;

the monomer at the selected site in the anti-sense stand includes a U (or a modified base which pairs with A), the corresponding monomer in the target is an A, and the sense strand is chosen from a monomer which will not pair or which will form a non-canonical pairing, e.g., U or G;

the monomer at the selected site in the anti-sense stand includes a C (or a modified base which pairs with G), the corresponding monomer in the target is a G, and the sense strand is chosen a monomer which will not pair or which will form a non-canonical pairing, e.g., G, A_{cis}, A_{trans}, or U; or

the monomer at the selected site in the anti-sense stand includes a G (or a modified base which pairs with C), the corresponding monomer in the target is a C, and the sense strand is chosen from a monomer which will not pair or which will form a non-canonical pairing.

In another embodiment a non-naturally occurring or modified monomer or monomers is chosen such that when it occupies complementary a position in an iRNA agent they exhibit a first free energy of dissociation and when one (or both) of them pairs with a naturally occurring monomer, the pair exhibits a second free energy of dissociation, which is usually higher than that

of the pairing of the first and second monomers. E.g., when the first and second monomers occupy complementary positions they either do not pair and have no substantial level of H-bonding, or form a weaker bond than one of them would form with a naturally occurring monomer, and reduce the stability of that duplex, but when the duplex dissociates at least one of the strands will form a duplex with a target in which the selected monomer will promote stability, e.g., the monomer will form a more stable pair with a naturally occurring monomer in the target sequence than the pairing it formed in the iRNA agent.

An example of such a pairing is 2-amino A and either of a 2-thio pyrimidine analog of U or T. As is discussed above, when placed in complementary positions of the iRNA agent these monomers will pair very poorly and will minimize stability. However, a duplex is formed between 2 amino A and the U of a naturally occurring target, or a duplex is formed between 2-thio U and the A of a naturally occurring target or 2-thio T and the A of a naturally occurring target will have a relatively higher free energy of dissociation and be more stable.

The monomer at the selected position in the sense strand can be a universal pairing moiety. A universal pairing agent will form some level of H bonding with more than one and preferably all other naturally occurring monomers. An examples of a universal pairing moiety is a monomer which includes 3-nitro pyrrole. Examples of other candidate universal base analogs can be found in the art, e.g., in Loakes, 2001, NAR 29: 2437-2447, hereby incorporated by reference. In these cases the monomer at the corresponding position of the anti-sense strand can be chosen for its ability to form a duplex with the target and can include, e.g., A, U, G, or C.

In another aspect, the invention features, an iRNA agent which includes:

a sense sequence, which preferably does not target a sequence in a subject, and an anti-sense sequence, which targets a plurality of target sequences in a subject, wherein the targets differ in sequence at only 1 or a small number, e.g., no more than 5, 4, 3 or 2 positions. The sense and anti-sense sequences have sufficient complementarity to each other to hybridize, e.g., under physiological conditions, e.g., under physiological conditions but not in contact with a helicase or other unwinding enzyme. In the sequence of the anti-sense strand of the iRNA agent is selected such that at one, some, or all of the positions which correspond to positions that differe in sequence between the target sequences, the anti-sense strand will include a monomer which will form H-bonds with at least two different target sequences. In a preferred example the anti-sense sequence will include a universal or promiscuous monomer, e.g., a monomer which

includes 5-nitro pyrrole, 2-amino A, 2-thio U or 2-thio T, or other universal base referred to herein.

In a preferred embodiment the iRNA agent targets repeated sequences (which differ at only one or a small number of positions from each other) in a single gene, a plurality of genes, or a viral genome, e.g., the HCV genome.

An embodiment is illustrated in the FIGs. 2 and 3.

In another aspect, the invention features, determining, e.g., by measurement or calculation, the stability of a pairing between monomers at a selected or constrained positoin in the iRNA agent duplex, and preferably determining the stability for the corresponding pairing in a duplex between a sequence form the iRNA agent and another RNA, e.g., a taret sequence. The determinations can be compared. An iRNA agent thus analysed can be used in the devolopement of a further modified iRNA agent or can be administered to a subject. This analysis can be performed successively to refine or desing optimized iRNA agents.

In another aspect, the invention features, a kit which inlcudes one or more of the folowing an iRNA described herein, a sterile container in which the iRNA agent is discolsed, and instructions for use.

In another aspect, the invention features, an iRNA agent containing a constrained sequence made by a method described herein. The iRNA agent can target one or more of the genes referred to herein.

iRNA agents having constrained or selected sites, e.g., as described herein, can be used in any way described herein. Accordingly, they iRNA agents having constrained or selected sites, e.g., as described herein, can be used to silence a target, e.g., in any of the methods described herein and to target any of the genes described herein or to treat any of the disorders described herein. iRNA agents having constrained or selected sites, e.g., as described herein, can be incorporated into any of the formulations or preparations, e.g., pharmaceutical or sterile preparations described herein. iRNA agents having constrained or selected sites, e.g., as described herein, can be administered by any of the routes of administration described herein.

The term "other than canonical Watson-Crick pairing" as used herein, refers to a pairing between a first monomer in a first sequence and a second monomer at the corresponding position in a second sequence of a duplex in which one or more of the following is true: (1) there is

essentially no pairing between the two, e.g., there is no significant level of H bonding between the monomers or binding between the monomers does not contribute in any significant way to the stability of the duplex; (2) the monomers are a non-canonical paring of monomers having a naturally occurring bases, i.e., they are other than A-T, A-U, or G-C, and they form monomer-monomer H bonds, although generally the H bonding pattern formed is less strong than the bonds formed by a canonical pairing; or(3) at least one of the monomers includes a non-naturally occurring bases and the H bonds formed between the monomers is, preferably formed is less strong than the bonds formed by a canonical pairing, namely one or more of A-T, A-U, G-C.

The term "off-target" as used herein, refers to as a sequence other than the sequence to be silenced.

Universal Bases: "wild-cards" ; shape-based complementarity

Bi-stranded, multisite replication of a base pair between difluorotoluene and adenine: confirmation by
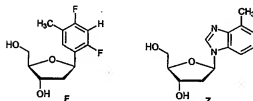
'inverse' sequencing. Liu, D.; Moran, S.; Kool, E. T. *Chem. Biol.*, 1997, *4*, 919-926)

(Importance of terminal base pair hydrogen-bonding in 3'-end proofreading by the Klenow fragment of DNA polymerase I. Morales, J. C.; Kool, E. T. *Biochemistry*, 2000, *39*, 2626-2632)

(Selective and stable DNA base pairing without hydrogen bonds. Matray, T, J.; Kool, E. T. *J. Am. Chem. Soc.*, 1998, *120*, 6191-6192)
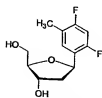
(Difluorotoluene, a nonpolar isostere for thymine, codes specifically and efficiently for adenine in DNA replication. Moran, S. Ren, R. X.-F.; Rumney IV, S.; Kool, E. T. *J. Am. Chem. Soc.*, 1997, *119*, 2056-2057)

(Structure and base pairing properties of a replicable nonpolar isostere for deoxyadenosine. Guckian, K. M.; Morales, J. C.; Kool, E. T. *J. Org. Chem.*, 1998, *63*, 9652-9656)

NO2

HO

OH
3-nitropyrrole

HO — NO₂

OH
5-nitroindole

MICS

PIM

5MICS
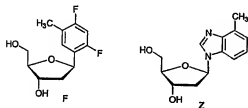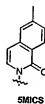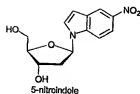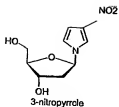
(Universal bases for hybridization, replication and chain termination. Berger, M.; Wu. Y.; Ogawa, A. K.;
McMinn, D. L.; Schultz, P.G.; Romesberg, F. E. *Nucleic Acids Res.*, 2000, *28*, 2911-2914)

TM

DM

ICS

PICS

2MN

DMN

7AI

2Np

3MN

(1. Efforts toward the expansion of the genetic alphabet: Information storage and replication with unnatural
hydrophobic base pairs. Ogawa, A. K.; Wu; McMinn, D. L.; Liu, J.; Schultz, P. G.; Romesberg, F. E. *J. Am.
Chem. Soc.*, 2000, *122*, 3274-3287. 2. Rational design of an unnatural base pair with increased kinetic
selectivity. Ogawa, A. K.; Wu. Y.; Berger, M.; Schultz, P. G.; Romesberg, F. E. *J. Am. Chem. Soc.*, 2000, *122*,
8803-8804)

7AI

(Efforts toward expansion of the genetic alphabet: replication of DNA with three base pairs. Tae, E. L.; Wu, Y.; Xia, G.; Schultz, P. G.; Romesberg, F. E. *J. Am. Chem. Soc.*, 2001, *123*, 7439-7440)
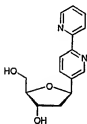
(1. Efforts toward expansion of the genetic alphabet: Optimization of interbase hydrophobic interactions. Wu, Y.; Ogawa, A. K.; Berger, M.; McMinn, D. L.; Schultz, P. G.; Romesberg, F. E. *J. Am. Chem. Soc.*, 2000, *122*, 7621-7632. 2. Efforts toward expansion of genetic alphabet: DNA polymerase recognition of a highly stable, self-pairing hydrophobic base. McMinn, D. L.; Ogawa. A. K.; Wu, Y.; Liu, J.; Schultz, P. G.; Romesberg, F. E. *J. Am. Chem. Soc.*, 1999, *121*, 11585-11586)

(A stable DNA duplex containing a non-hydrogen-bonding and non-shape complementary base couple: Interstrand stacking as the stability determining factor. Brotschi, C.; Haberli, A.; Leumann, C, J. *Angew. Chem. Int. Ed.*, 2001, *40*, 3012-3014)

(2,2'-Bipyridine Ligandoside: A novel building block for modifying DNA with intra-duplex metal complexes. Weizman, H.; Tor, Y. *J. Am. Chem. Soc.*, 2001, *123*, 3375-3376)

d2APy   d2APm
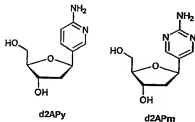
(Minor groove hydration is critical to the stability of DNA duplexes. Lan, T.; McLaughlin, L. W. *J. Am. Chem. Soc.*, 2000, *122*, 6512-13)
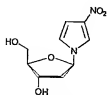
(Effect of the Universal base 3-nitropyrrole on the selectivity of neighboring natural bases. Oliver, J. S.; Parker, K. A.; Suggs, J. W. *Organic Lett.*, 2001, *3*, 1977-1980. 2. Effect of the 1-(2'-deoxy-β-D-ribofuranosyl)-3-nitropyrrol residue on the stability of DNA duplexes and triplexes. Amosova, O.; George J.; Fresco, J. R. *Nucleic Acids Res.*, 1997, *25*, 1930-1934. 3. Synthesis, structure and deoxyribonucleic acid sequencing with a universal nucleosides: 1-(2'-deoxy-β-D-ribofuranosyl)-3-nitropyrrole. Bergstrom, D. E.; Zhang, P.; Toma, P. H.; Andrews, P. C.; Nichols, R. *J. Am. Chem. Soc.*, 1995, *117*, 1201-1209)

(
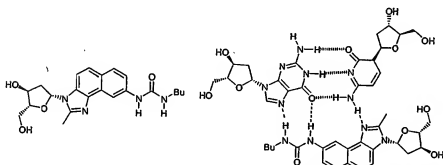
(Model studies directed toward a general triplex DNA recognition scheme: a novel DNA base that binds a CG base-pair in an organic solvent. Zimmerman, S. C.; Schmitt, P. *J. Am. Chem. Soc.*, 1995, *117*, 10769-10770)
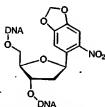
(A universal, photocleavable DNA base: nitropiperonyl 2'-deoxyriboside. *J. Org. Chem.*, 2001, *66*, 2067-2071)
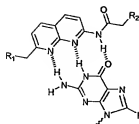
(Recognition of a single guanine bulge by 2-acylamino-1,8-naphthyridine. Nakatani, K.; Sando, S.; Saito, I. *J. Am. Chem. Soc.*, 2000, *122*, 2172-2177. b. Specific binding of 2-amino-1,8-naphthyridine into single guanine bulge as evidenced by photooxidation of GC doublet, Nakatani, K.; Sando, S.; Yoshida, K.; Saito, I. *Bioorg. Med. Chem. Lett.*, 2001, *11*, 335-337)
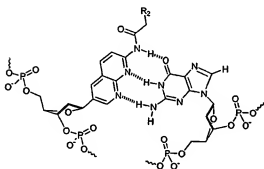
Other universal bases can have the following formulas:
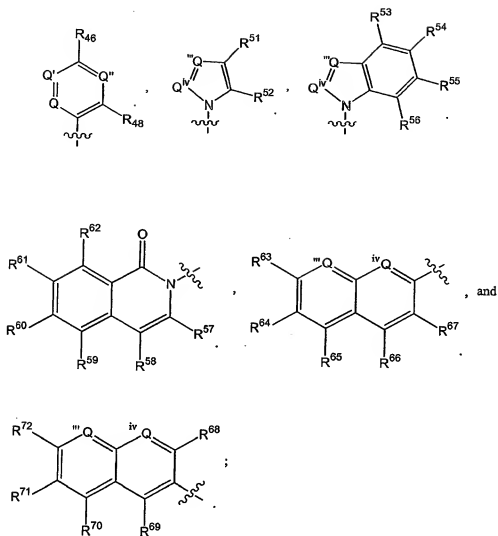
wherein:

Q is N or CR^{44};

Q' is N or CR^{45};

Q'' is N or CR^{47};

Q''' is N or CR^{49};

Q^{iv} is N or CR^{50};

R^{44} is hydrogen, halo, hydroxy, nitro, protected hydroxy, NH_2, NHR^b, or NR^bR^c, C_1-C_6 alkyl, C_6-C_{10} aryl, C_6-C_{10} heteroaryl, C_3-C_8 heterocyclyl, or when taken together with R^{45} forms $-OCH_2O-$;

R^{45} is hydrogen, halo, hydroxy, nitro, protected hydroxy, NH_2, NHR^b, or NR^bR^c, C_1-C_6 alkyl, C_6-C_{10} aryl, C_6-C_{10} heteroaryl, C_3-C_8 heterocyclyl, or when taken together with R^{44} or R^{46} forms $-OCH_2O-$;

R^{46} is hydrogen, halo, hydroxy, nitro, protected hydroxy, NH_2, NHR^b, or NR^bR^c, C_1-C_6 alkyl, C_6-C_{10} aryl, C_6-C_{10} heteroaryl, C_3-C_8 heterocyclyl, or when taken together with R^{45} or R^{47} forms $-OCH_2O-$;

R^{47} is hydrogen, halo, hydroxy, nitro, protected hydroxy, NH_2, NHR^b, or NR^bR^c, C_1-C_6 alkyl, C_6-C_{10} aryl, C_6-C_{10} heteroaryl, C_3-C_8 heterocyclyl, or when taken together with R^{46} or R^{48} forms $-OCH2O-$;

R^{48} is hydrogen, halo, hydroxy, nitro, protected hydroxy, NH_2, NHR^b, or NR^bR^c, C_1-C_6 alkyl, C_6-C_{10} aryl, C_6-C_{10} heteroaryl, C_3-C_8 heterocyclyl, or when taken together with R^{47} forms $-OCH2O-$;

R^{49} R^{50}, R^{51}, R^{52}, R^{53}, R^{54}, R^{57}, R^{58}, R^{59}, R^{60}, R^{61}, R^{62}, R^{63}, R^{64}, R^{65}, R^{66}, R^{67}, R^{68}, R^{69}, R^{70}, R^{71}, and R^{72} are each independently selected from hydrogen, halo, hydroxy, nitro, protected hydroxy, NH_2, NHR^b, or NR^bR^c, C_1-C_6 alkyl, C_2-C_6 alkynyl, C_6-C_{10} aryl, C_6-C_{10} heteroaryl, C_3-C_8 heterocyclyl, $NC(O)R^{17}$, or $NC(O)R^o$;

R^{55} is hydrogen, halo, hydroxy, nitro, protected hydroxy, NH_2, NHR^b, or NR^bR^c, C_1-C_6 alkyl, C_2-C_6 alkynyl, C_6-C_{10} aryl, C_6-C_{10} heteroaryl, C_3-C_8 heterocyclyl, $NC(O)R^{17}$, or $NC(O)R^o$, or when taken together with R^{56} forms a fused aromatic ring which may be optionally substituted;

R^{56} is hydrogen, halo, hydroxy, nitro, protected hydroxy, NH_2, NHR^b, or NR^bR^c, C_1-C_6 alkyl, C_2-C_6 alkynyl, C_6-C_{10} aryl, C_6-C_{10} heteroaryl, C_3-C_8 heterocyclyl, $NC(O)R^{17}$, or $NC(O)R^o$, or when taken together with R^{55} forms a fused aromatic ring which may be optionally substituted;

R^{17} is halo, NH_2, NHR^b, or NR^bR^c;

R^b is C_1-C_6 alkyl or a nitrogen protecting group;

R^c is C_1-C_6 alkyl; and

R^o is alkyl optionally substituted with halo, hydroxy, nitro, protected hydroxy, NH_2, NHR^b, or NR^bR^c, C_1-C_6 alkyl, C_2-C_6 alkynyl, C_6-C_{10} aryl, C_6-C_{10} heteroaryl, C_3-C_8 heterocyclyl, $NC(O)R^{17}$, or $NC(O)R^o$.

5   Examples of universal bases include:
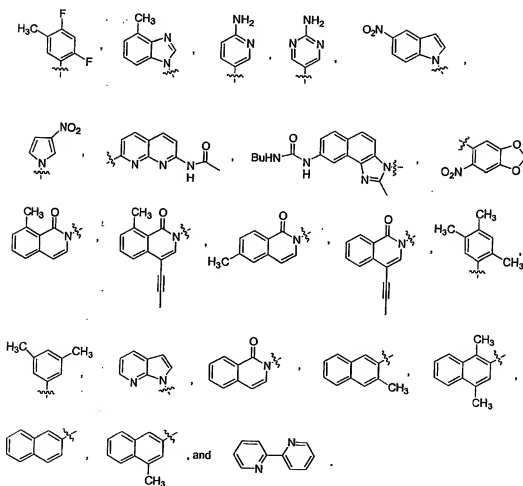
Asymmetrical Modifications

An RNA, e.g., an iRNA agent, can be asymmetrically modified as described herein, and as described in International Application Serial No. PCT/US04/07070, filed March 8, 2004, which is hereby incorporated by reference.

In addition, the invention includes iRNA agents having asymmetrical modifications and another element described herein. E.g., the invention includes an iRNA agent described herein, e.g., a palindromic iRNA agent, an iRNA agent having a non canonical pairing, an iRNA agent which targets a gene described herein, e.g., a gene active in the kidney, an iRNA agent having an architecture or structure described herein, an iRNA associated with an amphipathic delivery agent described herein, an iRNA associated with a drug delivery module described herein, an iRNA agent administered as described herein, or an iRNA agent formulated as described herein, which also incorporates an asymmetrical modification.

An asymmetrically modified iRNA agent is one in which a strand has a modification which is not present on the other strand. An asymmetrical modification is a modification found on one strand but not on the other strand. Any modification, e.g., any modification described herein, can be present as an asymmetrical modification. An asymmetrical modification can confer any of the desired properties associated with a modification, e.g., those properties discussed herein. E.g., an asymmetrical modification can: confer resistance to degradation, an alteration in half life; target the iRNA agent to a particular target, e.g., to a particular tissue; modulate, e.g., increase or decrease, the affinity of a strand for its complement or target sequence; or hinder or promote modification of a terminal moiety, e.g., modification by a kinase or other enzymes involved in the RISC mechanism pathway. The designation of a modification as having one property does not mean that it has no other property, e.g., a modification referred to as one which promotes stabilization might also enhance targeting.

While not wishing to be bound by theory or any particular mechanistic model, it is believed that asymmetrical modification allows an iRNA agent to be optimized in view of the different or "asymmetrical" functions of the sense and antisense strands. For example, both strands can be modified to increase nuclease resistance, however, since some changes can inhibit RISC activity, these changes can be chosen for the sense stand . In addition, since some modifications, e.g., targeting moieties, can add large bulky groups that, e.g., can interfere with the cleavage activity of the RISC complex, such modifications are preferably placed on the sense strand. Thus, targeting moieties, especially bulky ones (e.g. cholesterol), are preferentially added

to the sense strand. In one embodiment, an asymmetrical modification in which a phosphate of the backbone is substituted with S, e.g., a phosphorothioate modification, is present in the antisense strand, and a 2' modification, e.g., 2' OMe is present in the sense strand. A targeting moiety can be present at either (or both) the 5' or 3' end of the sense strand of the iRNA agent. In a preferred example, a P of the backbone is replaced with S in the antisense strand, 2'OMe is present in the sense strand, and a targeting moiety is added to either the 5' or 3' end of the sense strand of the iRNA agent.

In a preferred embodiment an asymmetrically modified iRNA agent has a modification on the sense strand which modification is not found on the antisense strand and the antisense strand has a modification which is not found on the sense strand.

Each strand can include one or more asymmetrical modifications. By way of example: one strand can include a first asymmetrical modification which confers a first property on the iRNA agent and the other strand can have a second asymmetrical modification which confers a second property on the iRNA. E.g., one strand, e.g., the sense strand can have a modification which targets the iRNA agent to a tissue, and the other strand, e.g., the antisense strand, has a modification which promotes hybridization with the target gene sequence.

In some embodiments both strands can be modified to optimize the same property, e.g., to increase resistance to nucleolytic degradation, but different modifications are chosen for the sense and the antisense strands, e.g., because the modifications affect other properties as well. E.g., since some changes can affect RISC activity these modifications are chosen for the sense strand.

In an embodiment one strand has an asymmetrical 2' modification, e.g., a 2' OMe modification, and the other strand has an asymmetrical modification of the phosphate backbone, e.g., a phosphorothioate modification. So, in one embodiment the antisense strand has an asymmetrical 2' OMe modification and the sense strand has an asymmetrical phosphorothioate modification (or vice versa). In a particularly preferred embodiment the RNAi agent will have asymmetrical 2'-O alkyl, preferably, 2'-OMe modifications on the sense strand and asymmetrical backbone P modification, preferably a phosphothioate modification in the antisense strand. There can be one or multiple 2'-OMe modifications, e.g., at least 2, 3, 4, 5, or 6, of the subunits of the sense strand can be so modified. There can be one or multiple phosphorothioate modifications, e.g., at least 2, 3, 4, 5, or 6, of the subunits of the antisense strand can be so modified. It is preferable to have an iRNA agent wherein there are multiple 2'-

OMe modifications on the sense strand and multiple phophorothioate modifications on the antisense strand. All of the subunits on one or both strands can be so modified. A particularly preferred embodiment of multiple asymmetric modification on both strands has a duplex region about 20-21, and preferably 19, subunits in length and one or two 3' overhangs of about 2 subunits in length.

Asymmetrical modifications are useful for promoting resistance to degradation by nucleases, e.g., endonucleases. iRNA agents can include one or more asymmetrical modifications which promote resistance to degradation. In preferred embodiments the modification on the antisense strand is one which will not interfere with silencing of the target, e.g., one which will not interfere with cleavage of the target. Most if not all sites on a strand are vulnerable, to some degree, to degradation by endonucleases. One can determine sites which are relatively vulnerable and insert asymmetrical modifications which inhibit degradation. It is often desirable to provide asymmetrical modification of a UA site in an iRNA agent, and in some cases it is desirable to provide the UA sequence on both strands with asymmetrical modification. Examples of modifications which inhibit endonucleolytic degradation can be found herein. Particularly favored modifications include: 2' modification, e.g., provision of a 2' OMe moiety on the U, especially on a sense strand; modification of the backbone, e.g., with the replacement of an O with an S, in the phosphate backbone, e.g., the provision of a phosphorothioate modification, on the U or the A or both, especially on an antisense strand; replacement of the U with a C5 amino linker; replacement of the A with a G (sequence changes are preferred to be located on the sense strand and not the antisense strand); and modification of the at the 2', 6', 7', or 8' position. Preferred embodiments are those in which one or more of these modifications are present on the sense but not the antisense strand, or embodiments where the antisense strand has fewer of such modifications.

Asymmetrical modification can be used to inhibit degradation by exonucleases. Asymmetrical modifications can include those in which only one strand is modified as well as those in which both are modified. In preferred embodiments the modification on the antisense strand is one which will not interfere with silencing of the target, e.g., one which will not interfere with cleavage of the target. Some embodiments will have an asymmetrical modification on the sense strand, e.g., in a 3' overhang, e.g., at the 3' terminus, and on the antisense strand, e.g., in a 3' overhang, e.g., at the 3' terminus. If the modifications introduce moieties of different size it is preferable that the larger be on the sense strand. If the

modifications introduce moieties of different charge it is preferable that the one with greater charge be on the sense strand.

Examples of modifications which inhibit exonucleolytic degradation can be found herein. Particularly favored modifications include: 2' modification, e.g., provision of a 2' OMe moiety in a 3' overhang, e.g., at the 3' terminus (3' terminus means at the 3' atom of the molecule or at the most 3' moiety, e.g., the most 3' P or 2' position, as indicated by the context); modification of the backbone, e.g., with the replacement of a P with an S, e.g., the provision of a phosphorothioate modification, or the use of a methylated P in a 3' overhang, e.g., at the 3' terminus; combination of a 2' modification, e.g., provision of a 2' O Me moiety and modification of the backbone, e.g., with the replacement of a P with an S, e.g., the provision of a phosphorothioate modification, or the use of a methylated P, in a 3' overhang, e.g., at the 3' terminus; modification with a 3' alkyl; modification with an abasic pyrolidine in a 3' overhang, e.g., at the 3' terminus; modification with naproxene, ibuprofen, or other moieties which inhibit degradation at the 3' terminus. Preferred embodiments are those in which one or more of these modifications are present on the sense but not the antisense strand, or embodiments where the antisense strand has fewer of such modifications.

Modifications, e.g., those described herein, which affect targeting can be provided as asymmetrical modifications. Targeting modifications which can inhibit silencing, e.g., by inhibiting cleavage of a target, can be provided as asymmetrical modifications of the sense strand. A biodistribution altering moiety, e.g., cholesterol, can be provided in one or more, e.g., two, asymmetrical modifications of the sense strand. Targeting modifications which introduce moieties having a relatively large molecular weight, e.g., a molecular weight of more than 400, 500, or 1000 daltons, or which introduce a charged moiety (e.g., having more than one positive charge or one negative charge) can be placed on the sense strand.

Modifications, e.g., those described herein, which modulate, e.g., increase or decrease, the affinity of a strand for its compliment or target, can be provided as asymmetrical modifications. These include: 5 methyl U; 5 methyl C; pseudouridine, Locked nucleic acids ,2 thio U and 2-amino-A. In some embodiments one or more of these is provided on the antisense strand.

iRNA agents have a defined structure, with a sense strand and an antisense strand, and in many cases short single strand overhangs, e.g., of 2 or 3 nucleotides are present at one or both 3' ends. Asymmetrical modification can be used to optimize the activity of such a structure, e.g.,

by being placed selectively within the iRNA. E.g., the end region of the iRNA agent defined by the 5' end of the sense strand and the 3' end of the antisense strand is important for function. This region can include the terminal 2, 3, or 4 paired nucleotides and any 3' overhang. In preferred embodiments asymmetrical modifications which result in one or more of the following are used: modifications of the 5' end of the sense strand which inhibit kinase activation of the sense strand, including, e.g., attachments of conjugates which target the molecule or the use modifications which protect against 5' exonucleolytic degradation; or modifications of either strand, but preferably the sense strand, which enhance binding between the sense and antisense strand and thereby promote a "tight" structure at this end of the molecule.

The end region of the iRNA agent defined by the 3' end of the sense strand and the 5' end of the antisense strand is also important for function. This region can include the terminal 2, 3, or 4 paired nucleotides and any 3' overhang. Preferred embodiments include asymmetrical modifications of either strand, but preferably the sense strand, which decrease binding between the sense and antisense strand and thereby promote an "open" structure at this end of the molecule. Such modifications include placing conjugates which target the molecule or modifications which promote nuclease resistance on the sense strand in this region. Modification of the antisense strand which inhibit kinase activation are avoided in preferred embodiments.

Exemplary modifications for asymmetrical placement in the sense strand include the following:

(a) backbone modifications, e.g., modification of a backbone P, including replacement of P with S, or P substituted with alkyl or allyl, e.g., Me, and dithioates (S-P=S); these modifications can be used to promote nuclease resistance;

(b) 2'-O alkyl, e.g., 2'-OMe, 3'-O alkyl, e.g., 3'-OMe (at terminal and/or internal positions); these modifications can be used to promote nuclease resistance or to enhance binding of the sense to the antisense strand, the 3' modifications can be used at the 5' end of the sense strand to avoid sense strand activation by RISC;

(c) 2'-5' linkages (with 2'-H, 2'-OH and 2'-OMe and with P=O or P=S) these modifications can be used to promote nuclease resistance or to inhibit binding of the sense to the antisense strand, or can be used at the 5' end of the sense strand to avoid sense strand activation by RISC;

(d) L sugars (e.g., L ribose, L-arabinose with 2'-H, 2'-OH and 2'-OMe); these modifications can be used to promote nuclease resistance or to inhibit binding of the sense to the antisense strand, or can be used at the 5' end of the sense strand to avoid sense strand activation by RISC;

(e) modified sugars (e.g., locked nucleic acids (LNA's), hexose nucleic acids (HNA's) and cyclohexene nucleic acids (CeNA's)); these modifications can be used to promote nuclease resistance or to inhibit binding of the sense to the antisense strand, or can be used at the 5' end of the sense strand to avoid sense strand activation by RISC;

(f) nucleobase modifications (e.g., C-5 modified pyrimidines, N-2 modified purines, N-7 modified purines, N-6 modified purines), these modifications can be used to promote nuclease resistance or to enhance binding of the sense to the antisense strand;

(g) cationic groups and Zwitterionic groups (preferably at a terminus), these modifications can be used to promote nuclease resistance;

(h) conjugate groups (preferably at terminal positions), e,g., naproxen, biotin, cholesterol, ibuprofen, folic acid, peptides, and carbohydrates; these modifications can be used to promote nuclease resistance or to target the molecule, or can be used at the 5' end of the sense strand to avoid sense strand activation by RISC.

Exemplary modifications for asymmetrical placement in the antisense strand include the following:

(a) backbone modifications, e.g., modification of a backbone P, including replacement of P with S, or P substituted with alkyl or allyl, e.g., Me, and dithioates (S-P=S);

(b) 2'-O alkyl, e.g., 2'-OMe, (at terminal positions);

(c) 2'-5' linkages (with 2'-H, 2'-OH and 2'-OMe) e.g., terminal at the 3' end); e.g., with P=O or P=S preferably at the 3'-end, these modifications are preferably excluded from the 5' end region as they may interfere with RISC enzyme activity such as kinase activity;

(d) L sugars (e.g, L ribose, L-arabinose with 2'-H, 2'-OH and 2'-OMe); e.g., terminal at the 3' end; e.g., with P=O or P=S preferably at the 3'-end, these modifications are preferably excluded from the 5' end region as they may interfere with kinase activity;

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(e) modified sugars (e.g., LNA's, HNA's and CeNA's); these modifications are preferably excluded from the 5' end region as they may contribute to unwanted enhancements of paring between the sense and antisense strands, it is often preferred to have a "loose" structure in the 5' region, additionally, they may interfere with kinase activity;

(f) nucleobase modifications (e.g., C-5 modified pyrimidines, N-2 modified purines, N-7 modified purines, N-6 modified purines);

(g) cationic groups and Zwitterionic groups (preferably at a terminus);

cationic groups and Zwitterionic groups at 2'-position of sugar; 3'-position of the sugar; as nucleobase modifications (e.g., C-5 modified pyrimidines, N-2 modified purines, N-7 modified purines, N-6 modified purines);

conjugate groups (preferably at terminal positions), e,g., naproxen, biotin, cholesterol, ibuprofen, folic acid, peptides, and carbohydrates, but bulky groups or generally groups which inhibit RISC activity should are less preferred.

The 5'-OH of the antisense strand should be kept free to promote activity. In some preferred embodiments modifications that promote nuclease resistance should be included at the 3' end, particularly in the 3' overhang.

In another aspect, the invention features a method of optimizing, e.g., stabilizing, an iRNA agent. The method includes selecting a sequence having activity, introducing one or more asymmetric modifications into the sequence, wherein the introduction of the asymmetric modification optimizes a property of the iRNA agent but does not result in a decrease in activity.

The decrease in activity can be less than a preselected level of decrease. In preferred embodiments decrease in activity means a decrease of less than 5, 10, 20, 40, or 50 % activity, as compared with an otherwise similar iRNA lacking the introduced modification. Activity can, e.g., be measured in vivo, or in vitro, with a result in either being sufficient to demonstrate the required maintenance of activity.

The optimized property can be any property described herein and in particular the properties discussed in the section on asymmetrical modifications provided herein. The modification can be any asymmetrical modification, e.g., an asymmetric modification described

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in the section on asymmetrical modifications described herein. Particularly preferred asymmetric modifications are 2'-O alkyl modifications, e.g., 2'-OMe modifications, particularly in the sense sequence, and modifications of a backbone O, particularly phosphorothioate modifications, in the antisense sequence.

In a preferred embodiment a sense sequence is selected and provided with an asymmetrical modification, while in other embodiments an antisense sequence is selected and provided with an asymmetrical modification. In some embodiments both sense and antisense sequences are selected and each provided with one or more asymmetrical modifications.

Multiple asymmetric modifications can be introduced into either or both of the sense and antisense sequence. A sequence can have at least 2, 4, 6, 8, or more modifications and all or substantially all of the monomers of a sequence can be modified.

Table 5 shows examples having strand I with a selected modification and strand II with a selected modification.

Table 5. Exemplary strand I- and strand II-modifications

Strand I	Strand II
Nuclease Resistance (e.g., 2'-OMe)	Biodistribution (e.g., P=S)
Biodistribution conjugate (e.g., Lipophile)	Protein Binding Functionality (e.g., Naproxen)
Tissue Distribution Functionality (e.g., Carbohydrates)	Cell Targeting Functionality (e.g., Folate for cancer cells)
Tissue Distribution Functionality (e.g., Kidney Cell Targetingmoieties)	Fusogenic Functionality (e.g., Polyethylene imines)
Cancer Cell Targeting (e.g., RGD peptides and imines)	Fusogenic Functionality (e.g., peptides)
Nuclease Resistance (e.g., 2'-OMe)	Increase in binding Affinity (5-Me-C, 5-Me-U, 2-thio-U, 2-amino-A, G-clamp, LNA)
Tissue Distribution Functionality	RISC activity improving Functionality
Helical conformation changing Functionalities	Tissue Distribution Functionality (P=S; lipophile, carbohydrates)

Z-X-Y Architecture

An RNA, e.g., an iRNA agent, can have a Z-X-Y architecture or structure such as those described herein and those described in copending, co-owned United States Provisional Application Serial No. 60/510,246, filed on October 9, 2003, which is hereby incorporated by reference, copending, co-owned United States Provisional Application Serial No. 60/510,318, filed on October 10, 2003, which is hereby incorporated by reference, and copending, co-owned International Application No. PCT/US04/07070, filed March 8, 2004.

In addition, the invention includes iRNA agents having a Z-X-Y structure and another element described herein. E.g., the invention includes an iRNA agent described herein, e.g., a palindromic iRNA agent, an iRNA agent having a non canonical pairing, an iRNA agent which targets a gene described herein, e.g., a gene active in the kidney, an iRNA associated with an amphipathic delivery agent described herein, an iRNA associated with a drug delivery module described herein, an iRNA agent administered as described herein, or an iRNA agent formulated as described herein, which also incorporates a Z-X-Y architecture.

Thus, an iRNA agent can have a first segment, the Z region, a second segment, the X region, and optionally a third region, the Y region:

$$Z—X—Y.$$

It may be desirable to modify subunits in one or both of Z and/or Y on one hand and X on the other hand. In some cases they will have the same modification or the same class of modification but it will more often be the case that the modifications made in Z and/or Y will differ from those made in X.

The Z region typically includes a terminus of an iRNA agent. The length of the Z region can vary, but will typically be from 2-14, more preferably 2-10, subunits in length. It typically is single stranded, i.e., it will not base pair with bases of another strand, though it may in some embodiments self associate, e.g., to form a loop structure. Such structures can be formed by the end of a strand looping back and forming an intrastrand duplex. E.g., 2, 3, 4, 5 or more intra-strand bases pairs can form, having a looped out or connecting region, typically of 2 or more

subunits which do not pair. This can occur at one or both ends of a strand. A typical embodiment of a Z region is a single strand overhang, e.g., an over hang of the length described elsewhere herein. The Z region can thus be or include a 3' or 5' terminal single strand. It can be sense or antisense strand but if it is antisense it is preferred that it is a 3- overhang. Typical inter-subunit bonds in the Z region include: P=O; P=S; S-P=S; P-NR$_2$; and P-BR$_2$. Chiral P=X, where X is S, N, or B) inter-subunit bonds can also be present. (These inter-subunit bonds are discussed in more detail elsewhere herein.) Other preferred Z region subunit modifications (also discussed elsewhere herein) can include: 3'-OR, 3'SR, 2'-OMe, 3'-OMe, and 2'OH modifications and moieties; alpha configuration bases; and 2' arabino modifications.

The X region will in most cases be duplexed, in the case of a single strand iRNA agent, with a corresponding region of the single strand, or in the case of a double stranded iRNA agent, with the corresponding region of the other strand. The length of the X region can vary but will typically be between 10-45 and more preferably between 15 and 35 subunits. Particularly preferred region X's will include 17, 18, 19, 29, 21, 22, 23, 24, or 25 nucleotide pairs, though other suitable lengths are described elsewhere herein and can be used. Typical X region subunits include 2'-OH subunits. In typical embodiments phosphate inter-subunit bonds are preferred while phophorothioate or non-phosphate bonds are absent. Other modifications preferred in the X region include: modifications to improve binding, e.g., nucleobase modifications; cationic nucleobase modifications; and C-5 modified pyrimidines, e.g., allylamines. Some embodiments have 4 or more consecutive 2'OH subunits. While the use of phosphorothioate is sometimes non preferred they can be used if they connect less than 4 consecutive 2'OH subunits.

The Y region will generally conform to the the parameters set out for the Z regions. However, the X and Z regions need not be the same, different types and numbers of modifications can be present, and infact, one will usually be a 3' overhang and one will usually be a 5' overhang.

In a preferred embodiment the iRNA agent will have a Y and/or Z region each having ribonucleosides in which the 2'-OH is substituted, e.g., with 2'-OMe or other alkyl; and an X region that includes at least four consecutive ribonucleoside subunits in which the 2'-OH remains unsubstituted.

The subunit linkages (the linkages between subunits) of an iRNA agent can be modified, e.g., to promote resistance to degradation. Numerous examples of such modifications are disclosed herein, one example of which is the phosphorothioate linkage. These modifications

can be provided bewteen the subunits of any of the regions, Y, X, and Z. However, it is preferred that their occureceis minimized and in particular it is preferred that consecutive modified linkages be avoided.

In a preferred embodiment the iRNA agent will have a Y and Z region each having ribonucleosides in which the 2'-OH is substituted, e.g., with 2'-OMe; and an X region that includes at least four consecutive subunits, e.g., ribonucleoside subunits in which the 2'-OH remains unsubstituted.

As mentioned above, the subunit linkages of an iRNA agent can be modified, e.g., to promote resistance to degradation. These modifications can be provided between the subunits of any of the regions, Y, X, and Z. However, it is preferred that they are minimized and in particular it is preferred that consecutive modified linkages be avoided.

Thus, in a preferred embodiment, not all of the subunit linkages of the iRNA agent are modified and more preferably the maximum number of consecutive subunits linked by other than a phospodiester bond will be 2, 3, or 4. Particulary preferred iRNA agents will not have four or more consecutive subunits, e.g., 2'-hydroxyl ribonucleoside subunits, in which each subunits is joined by modified linkages – i.e. linkages that have been modified to stabilize them from degradation as compared to the phosphodiester linkages that naturally occur in RNA and DNA.

It is particularly preferred to minimize the occurrence in region X. Thus, in preferred embodiments each of the nucleoside subunit linkages in X will be phosphodiester linkages, or if subunit linkages in region X are modified, such modifications will be minimized. E.g., although the Y and/or Z regions can include inter subunit linkages which have been stabilized against degradation, such modifications will be minimized in the X region, and in particular consecutive modifications will be minimized. Thus, in preferred embodiments the maximum number of consecutive subunits linked by other than a phospodiester bond will be 2, 3, or 4. Particulary preferred X regions will not have four or more consecutive subunits, e.g., 2'-hydroxyl ribonucleoside subunits, in which each subunits is joined by modified linkages – i.e. linkages that have been modified to stabilize them from degradation as compared to the phosphodiester linkages that naturally occur in RNA and DNA.

In a preferred embodiment Y and /or Z will be free of phosphorothioate linkages, though either or both may contain other modifications, e.g., other modifications of the subunit linkages.

In a preferred embodiment region X, or in some cases, the entire iRNA agent, has no more than 3 or no more than 4 subunits having identical 2' moieties.

In a preferred embodiment region X, or in some cases, the entire iRNA agent, has no more than 3 or no more than 4 subunits having identical subunit linkages.

In a preferred embodiment one or more phosphorothioate linkages (or other modifications of the subunit linkage) are present in Y and/or Z, but such modified linkages do not connect two adjacent subunits, e.g., nucleosides, having a 2' modification, e.g., a 2'-O-alkyl moiety. E.g., any adjacent 2'-O-alkyl moieties in the Y and/or Z, are connected by a linkage other than a a phosphorothioate linkage.

In a preferred embodiment each of Y and/or Z independently has only one phosphorothioate linkage between adjacent subunits, e.g., nucleosides, having a 2' modification, e.g., 2'-O-alkyl nucleosides. If there is a second set of adjacent subunits, e.g., nucleosides, having a 2' modification, e.g., 2'-O-alkyl nucleosides, in Y and/or Z that second set is connected by a linkage other than a phosphorothioate linkage, e.g., a modified linkage other than a phosphorothioate linkage.

In a prefered embodiment each of Y and/or Z independently has more than one phosphorothioate linkage connecting adjacent pairs of subunits, e.g., nucleosides, having a 2' modification, e.g., 2'-O-alkyl nucleosides, but at least one pair of adjacent subunits, e.g., nucleosides, having a 2' modification, e.g., 2'-O-alkyl nucleosides, are be connected by a linkage other than a phosphorothioate linkage, e.g., a modified linkage other than a phosphorothioate linkage.

In a prefered embodiment one of the above recited limitation on adjacent subunits in Y and or Z is combined with a limitation on the subunits in X. E.g., one or more phosphorothioate linkages (or other modifications of the subunit linkage) are present in Y and/or Z, but such modified linkages do not connect two adjacent subunits, e.g., nucleosides, having a 2' modification, e.g., a 2'-O-alkyl moiety. E.g., any adjacent 2'-O-alkyl moieties in the Y and/or Z, are connected by a linkage other than a a phosorothioate linkage. In addition, the X region has no more than 3 or no more than 4 identical subunits, e.g., subunits having identical 2' moieties or the X region has no more than 3 or no more than 4 subunits having identical subunit linkages.

A Y and/or Z region can include at least one, and preferably 2, 3 or 4 of a modification disclosed herein. Such modifications can be chosen, independently, from any modification

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described herein, e.g., from nuclease resistant subunits, subunits with modified bases, subunits with modified intersubunit linkages, subunits with modified sugars, and subunits linked to another moiety, e.g., a targeting moiety. In a preferred embodiment more than 1 of such subunits can be present but in some emobodiments it is prefered that no more than 1, 2, 3, or 4 of such modifications occur, or occur consecutively. In a preferred embodiment the frequency of the modification will differ between Y and /or Z and X, e.g., the modification will be present one of Y and/or Z or X and absent in the other.

An X region can include at least one, and preferably 2, 3 or 4 of a modification disclosed herein. Such modifications can be chosen, independently, from any modification desribed herein, e.g., from nuclease resistant subunits, subunits with modified bases, subunits with modified intersubunit linkages, subunits with modified sugars, and subunits linked to another moiety, e.g., a targeting moiety. In a preferred embodiment more than 1 of such subunits can b present but in some emobodiments it is prefered that no more than 1, 2, 3, or 4 of such modifications occur, or occur consecutively.

An RRMS (described elswhere herein) can be introduced at one or more points in one or both strands of a double-stranded iRNA agent. An RRMS can be placed in a Y and/or Z region, at or near (within 1, 2, or 3 positions) of the 3' or 5' end of the sense strand or at near (within 2 or 3 positions of) the 3' end of the antisense strand. In some embodiments it is prefered to not have an RRMS at or near (within 1, 2, or 3 positions of) the 5' end of the antisense strand. An RRMS can be positioned in the X region, and will preferably be positioned in the sense strand or in an area of the antisense strand not critical for antisense binding to the target.

Differential Modification of Terminal Duplex Stability

In one aspect, the invention features an iRNA agent which can have differential modification of terminal duplex stability (DMTDS).

In addition, the invention includes iRNA agents having DMTDS and another element described herein. E.g., the invention includes an iRNA agent described herein, e.g., a palindromic iRNA agent, an iRNA agent having a non canonical pairing, an iRNA agent which targets a gene described herein, e.g., a gene active in the kidney, an iRNA agent having an architecture or structure described herein, an iRNA associated with an amphipathic delivery agent described herein, an iRNA associated with a drug delivery module described herein, an

iRNA agent administered as described herein, or an iRNA agent formulated as described herein, which also incorporates DMTDS.

iRNA agents can be optimized by increasing the propensity of the duplex to disassociate or melt (decreasing the free energy of duplex association), in the region of the 5' end of the antisense strand duplex. This can be accomplished, e.g., by the inclusion of subunits which increase the propensity of the duplex to disassociate or melt in the region of the 5' end of the antisense strand. It can also be accomplished by the attachment of a ligand that increases the propensity of the duplex to disassociate of melt in the region of the 5' end . While not wishing to be bound by theory, the effect may be due to promoting the effect of an enzyme such as helicase, for example, promoting the effect of the enzyme in the proximity of the 5' end of the antisense strand.

The inventors have also discovered that iRNA agents can be optimized by decreasing the propensity of the duplex to disassociate or melt (increasing the free energy of duplex association), in the region of the 3' end of the antisense strand duplex. This can be accomplished, e.g., by the inclusion of subunits which decrease the propensity of the duplex to disassociate or melt in the region of the 3' end of the antisense strand. It can also be accomplished by the attachment of ligand that decreases the propensity of the duplex to disassociate of melt in the region of the 5' end.

Modifications which increase the tendency of the 5' end of the duplex to dissociate can be used alone or in combination with other modifications described herein, e.g., with modifications which decrease the tendency of the 3' end of the duplex to dissociate. Likewise, modifications which decrease the tendency of the 3' end of the duplex to dissociate can be used alone or in combination with other modifications described herein, e.g., with modifications which increase the tendency of the 5' end of the duplex to dissociate.

Decreasing the stability of the AS 5' end of the duplex

Subunit pairs can be ranked on the basis of their propensity to promote dissociation or melting (e.g., on the free energy of association or dissociation of a particular pairing, the simplest approach is to examine the pairs on an individual pair basis, though next neighbor or similar analysis can also be used). In terms of promoting dissociation:

A:U is preferred over G:C;

G:U is preferred over G:C;

I:C is preferred over G:C (I=inosine);

mismatches, e.g., non-canonical or other than canonical pairings (as described elsewhere herein) are preferred over canonical (A:T, A:U, G:C) pairings;

pairings which include a universal base are preferred over canonical pairings.

A typical ds iRNA agent can be diagrammed as follows:

S   5' $R_1 N_1 N_2 N_3 N_4 N_5$ [N] N_{-5} N_{-4} N_{-3} N_{-2} N_{-1} R_2 3'

AS   3' $R_3 N_1 N_2 N_3 N_4 N_5$ [N] N_{-5} N_{-4} N_{-3} N_{-2} N_{-1} R_4 5'

S:AS P_1 P_2 P_3 P_4 P_5 [N] $P_{-5} P_{-4} P_{-3} P_{-2} P_{-1}$ 5'

S indicates the sense strand; AS indicates antisense strand; R_1 indicates an optional (and nonpreferred) 5' sense strand overhang; R_2 indicates an optional (though preferred) 3' sense overhang; R_3 indicates an optional (though preferred) 3' antisense sense overhang; R_4 indicates an optional (and nonpreferred) 5' antisense overhang; N indicates subunits; [N] indicates that additional subunit pairs may be present; and P_x, indicates a paring of sense N_x and antisense N_x. Overhangs are not shown in the P diagram. In some embodiments a 3' AS overhang corresponds to region Z, the duplex region corresponds to region X, and the 3' S strand overhang corresponds to region Y, as described elsewhere herein. (The diagram is not meant to imply maximum or minimum lengths, on which guidance is provided elsewhere herein.)

It is preferred that pairings which decrease the propensity to form a duplex are used at 1 or more of the positions in the duplex at the 5' end of the AS strand. The terminal pair (the most 5' pair in terms of the AS strand) is designated as P_{-1}, and the subsequent pairing positions (going in the 3' direction in terms of the AS strand) in the duplex are designated, P_{-2}, P_{-3}, P_{-4}, P_{-5}, and so on. The preferred region in which to modify to modulate duplex formation is at P_{-5} through P_{-1}, more preferably P_{-4} through P_{-1}, more preferably P_{-3} through P_{-1}. Modification at P_{-1}, is particularly preferred, alone or with modification(s) other position(s), e.g., any of the positions just identified. It is preferred that at least 1, and more preferably 2, 3, 4, or 5 of the pairs of one of the recited regions be chosen independently from the group of:

A:U

G:U

I:C

mismatched pairs, e.g., non-canonical or other than canonical pairings or pairings which include a universal base.

In preferred embodiments the change in subunit needed to achieve a pairing which promotes dissociation will be made in the sense strand, though in some embodiments the change will be made in the antisense strand.

In a preferred embodiment the at least 2, or 3, of the pairs in P_{-1}, through P_{-4}, are pairs which promote disociation.

In a preferred embodiment the at least 2, or 3, of the pairs in P_{-1}, through P_{-4}, are A:U.

In a preferred embodiment the at least 2, or 3, of the pairs in P_{-1}, through P_{-4}, are G:U.

In a preferred embodiment the at least 2, or 3, of the pairs in P_{-1}, through P_{-4}, are I:C.

In a preferred embodiment the at least 2, or 3, of the pairs in P_{-1}, through P_{-4}, are mismatched pairs, e.g., non-canonical or other than canonical pairings pairings.

In a preferred embodiment the at least 2, or 3, of the pairs in P_{-1}, through P_{-4}, are pairings which include a universal base.

Increasing the stability of the AS 3' end of the duplex

Subunit pairs can be ranked on the basis of their propensity to promote stability and inhibit dissociation or melting (e.g., on the free energy of association or dissociation of a particular pairing, the simplest approach is to examine the pairs on an individual pair basis, though next neighbor or similar analysis can also be used). In terms of promoting duplex stability:

G:C   is preferred over A:U

Watson-Crick matches (A:T, A:U, G:C) are preferred over non-canonical or other than canonical pairings

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analogs that increase stability are preferred over Watson-Crick matches (A:T, A:U, G:C)

2-amino-A:U is preferred over A:U

2-thio U or 5 Me-thio-U:A are preferred over U:A

G-clamp (an analog of C having 4 hydrogen bonds):G is preferred over C:G

guanadinium-G-clamp:G is preferred over C:G

psuedo uridine:A is preferred over U:A

sugar modifications, e.g., 2' modifications, e.g., 2'F, ENA, or LNA, which enhance binding are preferred over non-modified moieties and can be present on one or both strands to enhance stability of the duplex. It is preferred that pairings which increase the propensity to form a duplex are used at 1 or more of the positions in the duplex at the 3' end of the AS strand. The terminal pair (the most 3' pair in terms of the AS strand) is designated as P_1, and the subsequent pairing positions (going in the 5' direction in terms of the AS strand) in the duplex are designated, P_2, P_3, P_4, P_5, and so on. The preferred region in which to modify to modulate duplex formation is at P_5 through P_1, more preferably P_4 through P_1, more preferably P_3 through P_1. Modification at P_1, is particularly preferred, alone or with mdification(s) at other position(s), e.g.,any of the positions just identified. It is preferred that at least 1, and more preferably 2, 3, 4, or 5 of the pairs of the recited regions be chosen independently from the group of:

G:C

a pair having an analog that increases stability over Watson-Crick matches (A:T, A:U, G:C)

2-amino-A:U

2-thio U or 5 Me-thio-U:A

G-clamp (an analog of C having 4 hydrogen bonds):G

guanadinium-G-clamp:G

psuedo uridine:A

a pair in which one or both subunits has a sugar modification, e.g., a 2' modification, e.g., 2'F, ENA, or LNA, which enhance binding.

In a preferred embodiment the least 2, or 3, of the pairs in P_{-1}, through P_{-4}, are pairs which promote duplex stability.

In a preferred embodiment the least 2, or 3, of the pairs in P_1, through P_4, are G:C.

In a preferred embodiment the least 2, or 3, of the pairs in P_1, through P_4, are a pair having an analog that increases stability over Watson-Crick matches.

In a preferred embodiment the least 2, or 3, of the pairs in P_1, through P_4, are 2-amino-A:U.

In a preferred embodiment the least 2, or 3, of the pairs in P_1, through P_4, are 2-thio U or 5 Me-thio-U:A.

In a preferred embodiment the least 2, or 3, of the pairs in P_1, through P_4, are G-clamp:G.

In a preferred embodiment the least 2, or 3, of the pairs in P_1, through P_4, are guanidinium-G-clamp:G.

In a preferred embodiment the least 2, or 3, of the pairs in P_1, through P_4, are psuedo uridine:A.

In a preferred embodiment the least 2, or 3, of the pairs in P_1, through P_4, are a pair in which one or both subunits has a sugar modification, e.g., a 2' modification, e.g., 2'F, ENA, or LNA, which enhances binding.

G-clamps and guanidinium G-clamps are discussed in the following references: Holmes and Gait, "The Synthesis of 2'-O-Methyl G-Clamp Containing Oligonucleotides and Their Inhibition of the HIV-1 Tat-TAR Interaction," Nucleosides, Nucleotides & Nucleic Acids, 22:1259-1262, 2003; Holmes et al., "Steric inhibition of human immunodeficiency virus type-1 Tat-dependent trans-activation in vitro and in cells by oligonucleotides containing 2'-O-methyl G-clamp ribonucleoside analogues," Nucleic Acids Research, 31:2759-2768, 2003; Wilds, et al., "Structural basis for recognition of guanosine by a synthetic tricyclic cytosine analogue:

Guanidinium G-clamp," Helvetica Chimica Acta, 86:966-978, 2003; Rajeev, *et al.*, "High-Affinity Peptide Nucleic Acid Oligomers Containing Tricyclic Cytosine Analogues," Organic Letters, 4:4395-4398, 2002; Ausin, *et al.*, "Synthesis of Amino- and Guanidino-G-Clamp PNA Monomers," Organic Letters, 4:4073-4075, 2002; Maier *et al.*, "Nuclease resistance of oligonucleotides containing the tricyclic cytosine analogues phenoxazine and 9-(2-aminoethoxy)-phenoxazine ("G-clamp") and origins of their nuclease resistance properties," Biochemistry, 41:1323-7, 2002; Flanagan, *et al.*, "A cytosine analog that confers enhanced potency to antisense oligonucleotides," Proceedings Of The National Academy Of Sciences Of The United States Of America, 96:3513-8, 1999.

<u>Simultaneously decreasing the stability of the AS 5'end of the duplex and increasing the stability of the AS 3' end of the duplex</u>

As is discussed above, an iRNA agent can be modified to both decrease the stability of the AS 5'end of the duplex and increase the stability of the AS 3' end of the duplex. This can be effected by combining one or more of the stability decreasing modifications in the AS 5' end of the duplex with one or more of the stability increasing modifications in the AS 3' end of the duplex. Accordingly a preferred embodiment includes modification in P_{-5} through P_{-1}, more preferably P_{-4} through P_{-1} and more preferably P_{-3} through P_{-1}. Modification at P_{-1}, is particularly preferred, alone or with other position, e.g., the positions just identified. It is preferred that at least 1, and more preferably 2, 3, 4, or 5 of the pairs of one of the recited regions of the AS 5' end of the duplex region be chosen independently from the group of:

A:U

G:U

I:C

mismatched pairs, e.g., non-canonical or other than canonical pairings which include a universal base; and

a modification in P_5 through P_1, more preferably P_4 through P_1 and more preferably P_3 through P_1. Modification at P_1, is particularly preferred, alone or with other position, e.g., the positions just identified. It is preferred that at least 1, and more preferably 2, 3, 4, or 5 of the pairs of one of the recited regions of the AS 3' end of the duplex region be chosen independently from the group of:

G:C

a pair having an analog that increases stability over Watson-Crick matches (A:T, A:U, G:C)

2-amino-A:U

2-thio U or 5 Me-thio-U:A

G-clamp (an analog of C having 4 hydrogen bonds):G

guanadinium-G-clamp:G

psuedo uridine:A

a pair in which one or both subunits has a sugar modification, e.g., a 2' modification, e.g., 2'F, ENA, or LNA, which enhance binding.

The invention also includes methods of selecting and making iRNA agents having DMTDS. E.g., when screening a target sequence for candidate sequences for use as iRNA agents one can select sequences having a DMTDS property described herein or one which can be modified, preferably with as few changes as possible, especially to the

AS strand, to provide a desired level of DMTDS.

The invention also includes, providing a candidate iRNA agent sequence, and modifying at least one P in P_{-5} through P_{-1} and/or at least one P in P_5 through P_1 to provide a DMTDS iRNA agent.

DMTDS iRNA agents can be used in any method described herein, e.g., to silence any gene disclosed herein, to treat any disorder described herein, in any formulation described herein, and generally in and/or with the methods and compositions described elsewhere herein. DMTDS iRNA agents can incorporate other modifications described herein, e.g., the attachment of targeting agents or the inclusion of modifications which enhance stability, e.g., the inclusion of nuclease resistant monomers or the inclusion of single strand overhangs (e.g., 3' AS overhangs and/or 3' S strand overhangs) which self associate to form intrastrand duplex structure.

Preferably these iRNA agents will have an architecture described herein.

Other Embodiments

An RNA, e.g., an iRNA agent, can be produced in a cell *in vivo*, e.g., from exogenous DNA templates that are delivered into the cell. For example, the DNA templates can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (U.S. Pat. No. 5,328,470), or by stereotactic injection (see, e.g., Chen *et al.*, *Proc. Natl. Acad. Sci. USA* 91:3054-3057, 1994). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. The DNA templates, for example, can include two transcription units, one that produces a transcript that includes the top strand of an iRNA agent and one that produces a transcript that includes the bottom strand of an iRNA agent. When the templates are transcribed, the iRNA agent is produced, and processed into sRNA agent fragments that mediate gene silencing.

In vivo Delivery

An iRNA agent can be linked, e.g., noncovalently linked to a polymer for the efficient delivery of the iRNA agent to a subject, e.g., a mammal, such as a human. The iRNA agent can, for example, be complexed with cyclodextrin. Cyclodextrins have been used as delivery vehicles of therapeutic compounds. Cyclodextrins can form inclusion complexes with drugs that are able to fit into the hydrophobic cavity of the cyclodextrin. In other examples, cyclodextrins form non-covalent associations with other biologically active molecules such as oligonucleotides and derivatives thereof. The use of cyclodextrins creates a water-soluble drug delivery complex, that can be modified with targeting or other functional groups. Cyclodextrin cellular delivery system for oligonucleotides described in U.S. Pat. No. 5,691,316, which is hereby incorporated by reference, are suitable for use in methods of the invention. In this system, an oligonucleotide is noncovalently complexed with a cyclodextrin, or the oligonucleotide is covalently bound to adamantine which in turn is non-covalently associated with a cyclodextrin.

The delivery molecule can include a linear cyclodextrin copolymer or a linear oxidized cyclodextrin copolymer having at least one ligand bound to the cyclodextrin copolymer. Delivery systems , as described in U.S. Patent No. 6,509,323, herein incorporated by reference, are suitable for use in methods of the invention. An iRNA agent can be bound to the linear cyclodextrin copolymer and/or a linear oxidized cyclodextrin copolymer. Either or both of the

cyclodextrin or oxidized cyclodextrin copolymers can be crosslinked to another polymer and/or bound to a ligand.

A composition for iRNA delivery can employ an "inclusion complex," a molecular compound having the characteristic structure of an adduct. In this structure, the "host

5   molecule" spatially encloses at least part of another compound in the delivery vehicle. The enclosed compound (the "guest molecule") is situated in the cavity of the host molecule without affecting the framework structure of the host. A "host" is preferably cyclodextrin, but can be any of the molecules suggested in U.S. Patent Publ. 2003/0008818, herein incorporated by reference.

Cyclodextrins can interact with a variety of ionic and molecular species, and the resulting
10   inclusion compounds belong to the class of "host-guest" complexes. Within the host-guest relationship, the binding sites of the host and guest molecules should be complementary in the stereoelectronic sense. A composition of the invention can contain at least one polymer and at least one therapeutic agent, generally in the form of a particulate composite of the polymer and therapeutic agent, e.g., the iRNA agent. The iRNA agent can contain one or more complexing

15   agents. At least one polymer of the particulate composite can interact with the complexing agent in a host-guest or a guest-host interaction to form an inclusion complex between the polymer and the complexing agent. The polymer and, more particularly, the complexing agent can be used to introduce functionality into the composition. For example, at least one polymer of the particulate composite has host functionality and forms an inclusion complex with a complexing agent

20   having guest functionality. Alternatively, at least one polymer of the particulate composite has guest functionality and forms an inclusion complex with a complexing agent having host functionality. A polymer of the particulate composite can also contain both host and guest functionalities and form inclusion complexes with guest complexing agents and host complexing agents. A polymer with functionality can, for example, facilitate cell targeting and/or cell

25   contact (e.g., targeting or contact to a kidney cell), intercellular trafficking, and/or cell entry and release.

Upon forming the particulate composite, the iRNA agent may or may not retain its biological or therapeutic activity. Upon release from the therapeutic composition, specifically, from the polymer of the particulate composite, the activity of the iRNA agent is restored. Accordingly, the particulate composite advantageously affords the iRNA agent protection against loss of activity due to, for example, degradation and offers enhanced bioavailability. Thus, a composition may be used to provide stability, particularly storage or solution stability, to

an iRNA agent or any active chemical compound. The iRNA agent may be further modified with a ligand prior to or after particulate composite or therapeutic composition formation. The ligand can provide further functionality. For example, the ligand can be a targeting moiety.

Physiological Effects

The iRNA agents described herein can be designed such that determining therapeutic toxicity is made easier by the complementarity of the iRNA agent with both a human and a non-human animal sequence. By these methods, an iRNA agent can consist of a sequence that is fully complementary to a nucleic acid sequence from a human *and* a nucleic acid sequence from at least one non-human animal, *e.g.*, a non-human mammal, such as a rodent, ruminant or primate. For example, the non-human mammal can be a mouse, rat, dog, pig, goat, sheep, cow, monkey, Pan paniscus, Pan troglodytes, Macaca mulatto, or Cynomolgus monkey. The sequence of the iRNA agent could be complementary to sequences within homologous genes, *e.g.*, oncogenes or tumor suppressor genes, of the non-human mammal and the human. By determining the toxicity of the iRNA agent in the non-human mammal, one can extrapolate the toxicity of the iRNA agent in a human. For a more strenuous toxicity test, the iRNA agent can be complementary to a human and more than one, *e.g.*, two or three or more, non-human animals.

The methods described herein can be used to correlate any physiological effect of an iRNA agent on a human, *e.g.*, any unwanted effect, such as a toxic effect, or any positive, or desired effect.

Delivery Module

An RNA, *e.g.*, an iRNA agent described herein, can be used with a drug delivery conjugate or module, such as those described herein and those described in copending, co-owned United States Provisional Application Serial No. 60/454,265, filed on March 12, 2003, and International Application Serial No. PCT/US04/07070, filed March 8, 2004, both of which are hereby incorporated by reference.

In addition, the invention includes iRNA agents described herein, *e.g.*, a palindromic iRNA agent, an iRNA agent hving a non canonical pairing, an iRNA agent which targets a gene described herein, *e.g.*, a gene active in the kidney, an iRNA agent having a chemical modification described herein, *e.g.*, a modification which enhances resistance to degradation, an iRNA agent having an architecture or structure described herein, an iRNA agent administered as

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described herein, or an iRNA agent formulated as described herein, combined with, associated with, and delivered by such a drug delivery conjugate or module.

The iRNA agents can be complexed to a delivery agent that features a modular complex. The complex can include a carrier agent linked to one or more of (preferably two or more, more preferably all three of): (a) a condensing agent (*e.g.*, an agent capable of attracting, *e.g.*, binding, a nucleic acid, *e.g.*, through ionic or electrostatic interactions); (b) a fusogenic agent (*e.g.*, an agent capable of fusing and/or being transported through a cell membrane, *e.g.*, an endosome membrane); and (c) a targeting group, *e.g.*, a cell or tissue targeting agent, *e.g.*, a lectin, glycoprotein, lipid or protein, *e.g.*, an antibody, that binds to a specified cell type such as a kidney cell.

An iRNA agent, *e.g.*, iRNA agent or sRNA agent described herein, can be linked, *e.g.*, coupled or bound, to the modular complex. The iRNA agent can interact with the condensing agent of the complex, and the complex can be used to deliver an iRNA agent to a cell, *e.g.*, *in vitro* or *in vivo*. For example, the complex can be used to deliver an iRNA agent to a subject in need thereof, *e.g.*, to deliver an iRNA agent to a subject having a disorder, *e.g.*, a disorder described herein, such as a disease or disorder of the kidney.

The fusogenic agent and the condensing agent can be different agents or the one and the same agent. For example, a polyamino chain, *e.g.*, polyethyleneimine (PEI), can be the fusogenic and/or the condensing agent.

The delivery agent can be a modular complex. For example, the complex can include a carrier agent linked to one or more of (preferably two or more, more preferably all three of):

(a) a condensing agent (*e.g.*, an agent capable of attracting, *e.g.*, binding, a nucleic acid, *e.g.*, through ionic interaction),

(b) a fusogenic agent (*e.g.*, an agent capable of fusing and/or being transported through a cell membrane, *e.g.*, an endosome membrane), and

(c) a targeting group, *e.g.*, a cell or tissue targeting agent, *e.g.*, a lectin, glycoprotein, lipid or protein, *e.g.*, an antibody, that binds to a specified cell type such as a kidney cell. A targeting group can be a thyrotropin, melanotropin, lectin, glycoprotein, surfactant protein A, Mucin carbohydrate, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-gulucosamine multivalent mannose, multivalent fucose, glycosylated polyaminoacids,

multivalent galactose, transferrin, bisphosphonate, polyglutamate, polyaspartate, a lipid,
cholesterol, a steroid, bile acid, folate, vitamin B12, biotin, Neproxin, or an RGD peptide or
RGD peptide mimetic.

Carrier agents

5   The carrier agent of a modular complex described herein can be a substrate for
attachment of one or more of: a condensing agent, a fusogenic agent, and a targeting group. The
carrier agent would preferably lack an endogenous enzymatic activity. The agent would
preferably be a biological molecule, preferably a macromolecule. Polymeric biological carriers
are preferred. It would also be preferred that the carrier molecule be biodegradable..

10   The carrier agent can be a naturally occurring substance, such as a protein (e.g., human
serum albumin (HSA), low-density lipoprotein (LDL), or globulin); carbohydrate (e.g., a
dextran, pullulan, chitin, chitosan, inulin, cyclodextrin or hyaluronic acid); or lipid. The carrier
molecule can also be a recombinant or synthetic molecule, such as a synthetic polymer, e.g., a
synthetic polyamino acid. Examples of polyamino acids include polylysine (PLL),
15   poly L-aspartic acid, poly L-glutamic acid, styrene-maleic acid anhydride copolymer, poly(L-
lactide-co-glycolied) copolymer, divinyl ether-maleic anhydride copolymer, N-(2-
hydroxypropyl)methacrylamide copolymer (HMPA), polyethylene glycol (PEG), polyvinyl
alcohol (PVA), polyurethane, poly(2-ethylacryllic acid), N-isopropylacrylamide polymers, or
polyphosphazine. Other useful carrier molecules can be identified by routine methods.

20   A carrier agent can be characterized by one or more of: (a) is at least 1 Da in size; (b) has
at least 5 charged groups, preferably between 5 and 5000 charged groups; (c) is present in the
complex at a ratio of at least 1:1 carrier agent to fusogenic agent; (d) is present in the complex at
a ratio of at least 1:1 carrier agent to condensing agent; (e) is present in the complex at a ratio of
at least 1:1 carrier agent to targeting agent.

Fusogenic agents

5   A fusogenic agent of a modular complex described herein can be an agent that is
responsive to, e.g., changes charge depending on, the pH environment. Upon encountering the
pH of an endosome, it can cause a physical change, e.g., a change in osmotic properties which
disrupts or increases the permeability of the endosome membrane. Preferably, the fusogenic
agent changes charge, e.g., becomes protonated, at pH lower than physiological range. For
example, the fusogenic agent can become protonated at pH 4.5-6.5. The fusogenic agent can

serve to release the iRNA agent into the cytoplasm of a cell after the complex is taken up, e.g., via endocytosis, by the cell, thereby increasing the cellular concentration of the iRNA agent in the cell.

In one embodiment, the fusogenic agent can have a moiety, e.g., an amino group, which, when exposed to a specified pH range, will undergo a change, e.g., in charge, e.g., protonation. The change in charge of the fusogenic agent can trigger a change, e.g., an osmotic change, in a vesicle, e.g., an endocytic vesicle, e.g., an endosome. For example, the fusogenic agent, upon being exposed to the pH environment of an endosome, will cause a solubility or osmotic change substantial enough to increase the porosity of (preferably, to rupture) the endosomal membrane.

The fusogenic agent can be a polymer, preferably a polyamino chain, e.g., polyethyleneimine (PEI). The PEI can be linear, branched, synthetic or natural. The PEI can be, e.g., alkyl substituted PEI, or lipid substituted PEI.

In other embodiments, the fusogenic agent can be polyhistidine, polyimidazole, polypyridine, polypropyleneimine, mellitin, or a polyacetal substance, e.g., a cationic polyacetal. In some embodiment, the fusogenic agent can have an alpha helical structure. The fusogenic agent can be a membrane disruptive agent, e.g., mellittin.

A fusogenic agent can have one or more of the following characteristics: (a) is at least 1Da in size; (b) has at least 10 charged groups, preferably between 10 and 5000 charged groups, more preferably between 50 and 1000 charged groups; (c) is present in the complex at a ratio of at least 1:1 fusogenic agent to carrier agent; (d) is present in the complex at a ratio of at least 1:1 fusogenic agent to condensing agent; (e) is present in the complex at a ratio of at least 1:1 fusogenic agent to targeting agent.

Other suitable fusogenic agents can be tested and identified by a skilled artisan. The ability of a compound to respond to, e.g., change charge depending on, the pH environment can be tested by routine methods, e.g., in a cellular assay. For example, a test compound is combined or contacted with a cell, and the cell is allowed to take up the test compound, e.g., by endocytosis. An endosome preparation can then be made from the contacted cells and the endosome preparation compared to an endosome preparation from control cells. A change, e.g., a decrease, in the endosome fraction from the contacted cell vs. the control cell indicates that the test compound can function as a fusogenic agent. Alternatively, the contacted cell and control cell can be evaluated, e.g., by microscopy, e.g., by light or electron microscopy, to determine a

difference in endosome population in the cells. The test compound can be labeled. In another type of assay, a modular complex described herein is constructed using one or more test or putative fusogenic agents. The modular complex can be constructed using a labeled nucleic acid instead of the iRNA. The ability of the fusogenic agent to respond to, e.g., change charge depending on, the pH environment, once the modular complex is taken up by the cell, can be evaluated, e.g., by preparation of an endosome preparation, or by microscopy techniques, as described above. A two-step assay can also be performed, wherein a first assay evaluates the ability of a test compound alone to respond to, e.g., change charge depending on, the pH environment; and a second assay evaluates the ability of a modular complex that includes the test compound to respond to, e.g., change charge depending on, the pH environment.

Condensing agent

The condensing agent of a modular complex described herein can interact with (e.g., attracts, holds, or binds to) an iRNA agent and act to (a) condense, e.g., reduce the size or charge of the iRNA agent and/or (b) protect the iRNA agent, e.g., protect the iRNA agent against degradation. The condensing agent can include a moiety, e.g., a charged moiety, that can interact with a nucleic acid, e.g., an iRNA agent, e.g., by ionic interactions. The condensing agent would preferably be a charged polymer, e.g., a polycationic chain. The condensing agent can be a polylysine (PLL), spermine, spermidine, polyamine, pseudopeptide-polyamine, peptidomimetic polyamine, dendrimer polyamine, arginine, amidine, protamine, cationic lipid, cationic porphyrin, quarternary salt of a polyamine, or an alpha helical peptide.

A condensing agent can have the following characteristics: (a) at least 1Da in size; (b) has at least 2 charged groups, preferably between 2 and 100 charged groups; (c) is present in the complex at a ratio of at least 1:1 condensing agent to carrier agent; (d) is present in the complex at a ratio of at least 1:1 condensing agent to fusogenic agent; (e) is present in the complex at a ratio of at least 1:1 condensing agent to targeting agent.

Other suitable condensing agents can be tested and identified by a skilled artisan, e.g., by evaluating the ability of a test agent to interact with a nucleic acid, e.g., an iRNA agent. The ability of a test agent to interact with a nucleic acid, e.g., an iRNA agent, e.g., to condense or protect the iRNA agent, can be evaluated by routine techniques. In one assay, a test agent is contacted with a nucleic acid, and the size and/or charge of the contacted nucleic acid is evaluated by a technique suitable to detect changes in molecular mass and/or charge. Such techniques include non-denaturing gel electrophoresis, immunological methods, e.g.,

immunoprecipitation, gel filtration, ionic interaction chromatography, and the like. A test agent is identified as a condensing agent if it changes the mass and/or charge (preferably both) of the contacted nucleic acid, compared to a control. A two-step assay can also be performed, wherein a first assay evaluates the ability of a test compound alone to interact with, e.g., bind to, e.g., condense the charge and/or mass of, a nucleic cid; and a second assay evaluates the ability of a modular complex that includes the test compound to interact with, e.g., bind to, e.g., condense the charge and/or mass of, a nucleic acid.

Amphipathic Delivery Agents

An RNA, e.g., an iRNA agent, described herein can be used with an amphipathic delivery conjugate or module, such as those described herein and those described in copending, co-owned United States Provisional Application Serial No. 60/455,050, filed on March 13, 2003, and International Application Serial No. PCT/US04/07070, filed March 8, 2004, which is hereby incorporated by reference.

In addition, the invention includes an iRNA agent described herein, e.g., a palindromic iRNA agent, an iRNA agent having a noncanonical pairing, an iRNA agent which targets a gene described herein, e.g., a gene active in the kidney, an iRNA agent having a chemical modification described herein, e.g., a modification which enhances resistance to degradation, an iRNA agent having an architecture or structure described herein, an iRNA agent administered as described herein, or an iRNA agent formulated as described herein, combined with, associated with, and delivered by such an amphipathic delivery conjugate.

An amphipathic molecule is a molecule having a hydrophobic and a hydrophilic region. Such molecules can interact with (e.g., penetrate or disrupt) lipids, e.g., a lipid bylayer of a cell. As such, they can serve as delivery agent for an associated (e.g., bound) iRNA (e.g., an iRNA or sRNA described herein). A preferred amphipathic molecule to be used in the compositions described herein (e.g., the amphipathic iRNA constructs descriebd herein) is a polymer. The polymer may have a secondary structure, e.g., a repeating secondary structure.

One example of an amphipathic polymer is an amphipathic polypeptide, e.g., a polypeptide having a secondary structure such that the polypeptide has a hydrophilic and a hybrophobic face. The design of amphipathic peptide structures (e.g., alpha-helical polypeptides) is routine to one of skill in the art. For example, the following references provide guidance: Grell et al. (2001) "Protein design and folding: template trapping of self-assembled helical

bundles" J Pept Sci 7(3):146-51; Chen et al. (2002) "Determination of stereochemistry stability coefficients of amino acid side-chains in an amphipathic alpha-helix" J Pept Res 59(1):18-33; Iwata et al. (1994) "Design and synthesis of amphipathic 3(10)-helical peptides and their interactions with phospholipid bilayers and ion channel formation" J Biol Chem 269(7):4928-33; Cornut et al. (1994) "The amphipathic alpha-helix concept. Application to the de novo design of ideally amphipathic Leu, Lys peptides with hemolytic activity higher than that of melittin" FEBS Lett 349(1):29-33; Negrete et al. (1998) "Deciphering the structural code for proteins: helical propensities in domain classes and statistical multiresidue information in alpha-helices," Protein Sci 7(6):1368-79.

Another example of an amphipathic polymer is a polymer made up of two or more amphipathic subunits, e.g., two or more subunits containing cyclic moieties (e.g., a cyclic moiety having one or more hydrophilic groups and one or more hydrophobic groups). For example, the subunit may contain a steroid, e.g., cholic acid; or a aromatic moiety. Such moieties preferably can exhibit atropisomerism, such that they can form opposing hydrophobic and hydrophilic faces when in a polymer structure.

The ability of a putative amphipathic molecule to interact with a lipid membrane, e.g., a cell membrane, can be tested by routine methods, e.g., in a cell free or cellular assay. For example, a test compound is combined or contacted with a synthetic lipid bilayer, a cellular membrane fraction, or a cell, and the test compound is evaluated for its ability to interact with, penetrate or disrupt the lipid bilayer, cell membrane or cell. The test compound can labeled in order to detect the interaction with the lipid bilayer, cell membrane or cell. In another type of assay, the test compound is linked to a reporter molecule or an iRNA agent (e.g., an iRNA or sRNA described herein) and the ability of the reporter molecule or iRNA agent to penetrate the lipid bilayer, cell membrane or cell is evaluated. A two-step assay can also be performed, wherein a first assay evaluates the ability of a test compound alone to interact with a lipid bilayer, cell membrane or cell; and a second assay evaluates the ability of a construct (e.g., a construct described herein) that includes the test compound and a reporter or iRNA agent to interact with a lipid bilayer, cell membrane or cell.

An amphipathic polymer useful in the compositions described herein has at least 2, preferably at least 5, more preferably at least 10, 25, 50, 100, 200, 500, 1000, 2000, 50000 or more subunits (e.g., amino acids or cyclic subunits). A single amphipathic polymer can be linked to one or more, e.g., 2, 3, 5, 10 or more iRNA agents (e.g., iRNA or sRNA agents

described herein). In some embodiments, an amphipathic polymer can contain both amino acid and cyclic subunits, e.g., aromatic subunits.

The invention features a composition that includes an iRNA agent (e.g., an iRNA or sRNA described herein) in association with an amphipathic molecule. Such compositions may be referred to herein as "amphipathic iRNA constructs." Such compositions and constructs are useful in the delivery or targeting of iRNA agents, e.g., delivery or targeting of iRNA agents to a cell. While not wanting to be bound by theory, such compositions and constructs can increase the porosity of, e.g., can penetrate or disrupt, a lipid (e.g., a lipid bilayer of a cell), e.g., to allow entry of the iRNA agent into a cell.

In one aspect, the invention relates to a composition comprising an iRNA agent (e.g., an iRNA or sRNA agent described herein) linked to an amphipathic molecule. The iRNA agent and the amphipathic molecule may be held in continuous contact with one another by either covalent or noncovalent linkages.

The amphipathic molecule of the composition or construct is preferably other than a phospholipid, e.g., other than a micelle, membrane or membrane fragment.

The amphipathic molecule of the composition or construct is preferably a polymer. The polymer may include two or more amphipathic subunits. One or more hydrophilic groups and one or more hydrophobic groups may be present on the polymer. The polymer may have a repeating secondary structure as well as a first face and a second face. The distribution of the hydrophilic groups and the hydrophobic groups along the repeating secondary structure can be such that one face of the polymer is a hydrophilic face and the other face of the polymer is a hydrophobic face.

The amphipathic molecule can be a polypeptide, e.g., a polypeptide comprising an α-helical conformation as its secondary structure.

In one embodiment, the amphipathic polymer includes one or more subunits containing one or more cyclic moiety (e.g., a cyclic moiety having one or more hydrophilic groups and/or one or more hydrophobic groups). In one embodiment, the polymer is a polymer of cyclic moieties such that the moieties have alternating hydrophobic and hydrophilic groups. For example, the subunit may contain a steroid, e.g., cholic acid. In another example, the subunit may contain an aromatic moiety. The aromatic moiety may be one that can exhibit

atropisomerism, e.g., a 2,2'-bis(substituted)-1-1'-binaphthyl or a 2,2'-bis(substituted) biphenyl.
A subunit may include an aromatic moiety of Formula (M):

(M)

5

The invention features a composition that includes iRNA agent (e.g., an iRNA or
sRNA described herein) in association with an amphipathic molecule. Such compositions may
be referred to herein as "amphipathic iRNA constructs." Such compositions and constructs are
useful in the delivery or targeting of iRNA agents, e.g., delivery or targeting of iRNA agents to a
10 cell. While not wanting to be bound by theory, such compositions and constructs can increase
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iRNA or sRNA agent described herein) linked to an amphipathic molecule. The iRNA agent and
15 the amphipathic molecule may be held in continuous contact with one another by either covalent
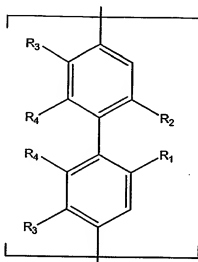or noncovalent linkages.

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phospholipid, e.g., other than a micelle, membrane or membrane fragment.

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In one embodiment, the amphipathic polymer includes one or more subunits containing one or more cyclic moiety (e.g., a cyclic moiety having one or more hydrophilic groups and/or one or more hydrophobic groups). In one embodiment, the polymer is a polymer of cyclic moieties such that the moieties have alternating hydrophobic and hydrophilic groups. For example, the subunit may contain a steroid, e.g., cholic acid. In another example, the subunit may contain an aromatic moiety. The aromatic moiety may be one that can exhibit atropisomerism, e.g., a 2,2'-bis(substituted)-1-1'-binaphthyl or a 2,2'-bis(substituted) biphenyl.

A subunit may include an aromatic moiety of Formula (M):

(M)

Referring to Formula M, R_1 is C_1-C_{100} alkyl optionally substituted with aryl, alkenyl, alkynyl, alkoxy or halo and/or optionally inserted with O, S, alkenyl or alkynyl; C_1-C_{100} perfluoroalkyl; or OR_5.

R_2 is hydroxy; nitro; sulfate; phosphate; phosphate ester; sulfonic acid; OR_6; or C_1-C_{100} alkyl optionally substituted with hydroxy, halo, nitro, aryl or alkyl sulfinyl, aryl or alkyl sulfonyl, sulfate, sulfonic acid, phosphate, phosphate ester, substituted or unsubstituted aryl, carboxyl, carboxylate, amino carbonyl, or alkoxycarbonyl, and/or optionally inserted with O, NH, S, S(O), SO_2, alkenyl, or alkynyl.

R_3 is hydrogen, or when taken together with R_4 froms a fused phenyl ring.

R_4 is hydrogen, or when taken together with R_3 froms a fused phenyl ring.

R_5 is C_1-C_{100} alkyl optionally substituted with aryl, alkenyl, alkynyl, alkoxy or halo and/or optionally inserted with O, S, alkenyl or alkynyl; or C_1-C_{100} perfluoroalkyl; and R_6 is C_1-C_{100} alkyl optionally substituted with hydroxy, halo, nitro, aryl or alkyl sulfinyl, aryl or alkyl sulfonyl, sulfate, sulfonic acid, phosphate, phosphate ester, substituted or unsubstituted aryl, carboxyl, carboxylate, amino carbonyl, or alkoxycarbonyl, and/or optionally inserted with O, NH, S, S(O), SO_2, alkenyl, or alkynyl.

Increasing cellular uptake of dsRNAs

A method of the invention that can include the administration of an iRNA agent and a drug that affects the uptake of the iRNA agent into the cell. The drug can be administered before, after, or at the same time that the iRNA agent is administered. The drug can be covalently linked to the iRNA agent. The drug can be, for example, a lipopolysaccharide, an activator of p38 MAP kinase, or an activator of NF-κB. The drug can have a transient effect on the cell.

The drug can increase the uptake of the iRNA agent into the cell, for example, by disrupting the cell's cytoskeleton, $e.g.$, by disrupting the cell's microtubules, microfilaments, and/or intermediate filaments. The drug can be, for example, taxon, vincristine, vinblastine, cytochalasin, nocodazole, japlakinolide, latrunculin A, phalloidin, swinholide A, indanocine, or myoservin.

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The drug can also increase the uptake of the iRNA agent into the cell by activating an inflammatory response, for example. Exemplary drug's that would have such an effect include tumor necrosis factor alpha (TNFalpha), interleukin-1 beta, or gamma interferon.

iRNA conjugates

An iRNA agent can be coupled, *e.g.*, covalently coupled, to a second agent. For example, an iRNA agent used to treat a particular disorder can be coupled to a second therapeutic agent, *e.g.*, an agent other than the iRNA agent. The second therapeutic agent is one which is directed to the treatment of the same disorder. For example, in the case of an iRNA used to treat a disorder characterized by unwanted cell proliferation, *e.g.*, cancer, the iRNA agent can be coupled to a second agent which has an anti-cancer effect. For example, it can be coupled to an agent which stimulates the immune system, *e.g.*, a CpG motif, or more generally an agent that activates a toll-like receptor and/or increases the production of gamma interferon.

iRNA Production

An iRNA can be produced, *e.g.*, in bulk, by a variety of methods. Exemplary methods include: organic synthesis and RNA cleavage, *e.g.*, *in vitro* cleavage.

Organic Synthesis

An iRNA can be made by separately synthesizing each respective strand of a double-stranded RNA molecule. The component strands can then be annealed.

A large bioreactor, *e.g.*, the OligoPilot II from Pharmacia Biotec AB (Uppsala Sweden), can be used to produce a large amount of a particular RNA strand for a given iRNA. The OligoPilotII reactor can efficiently couple a nucleotide using only a 1.5 molar excess of a phosphoramidite nucleotide. To make an RNA strand, ribonucleotides amidites are used. Standard cycles of monomer addition can be used to synthesize the 21 to 23 nucleotide strand for the iRNA. Typically, the two complementary strands are produced separately and then annealed, *e.g.*, after release from the solid support and deprotection.

Organic synthesis can be used to produce a discrete iRNA species. The complementary of the species to a particular target gene can be precisely specified. For example, the species may be complementary to a region that includes a polymorphism, *e.g.*, a single nucleotide polymorphism. Further the location of the polymorphism can be precisely defined. In some

embodiments, the polymorphism is located in an internal region, *e.g.*, at least 4, 5, 7, or 9 nucleotides from one or both of the termini.

dsRNA Cleavage

iRNAs can also be made by cleaving a larger ds iRNA. The cleavage can be mediated *in vitro* or *in vivo*. For example, to produce iRNAs by cleavage *in vitro*, the following method can be used:

In vitro transcription. dsRNA is produced by transcribing a nucleic acid (DNA) segment in both directions. For example, the HiScribe™ RNAi transcription kit (New England Biolabs) provides a vector and a method for producing a dsRNA for a nucleic acid segment that is cloned into the vector at a position flanked on either side by a T7 promoter. Separate templates are generated for T7 transcription of the two complementary strands for the dsRNA. The templates are transcribed *in vitro* by addition of T7 RNA polymerase and dsRNA is produced. Similar methods using PCR and/or other RNA polymerases (*e.g.*, T3 or SP6 polymerase) can also be used. In one embodiment, RNA generated by this method is carefully purified to remove endotoxins that may contaminate preparations of the recombinant enzymes.

In vitro cleavage. dsRNA is cleaved *in vitro* into iRNAs, for example, using a Dicer or comparable RNAse III-based activity. For example, the dsRNA can be incubated in an *in vitro* extract from Drosophila or using purified components, *e.g.* a purified RNAse or RISC complex (RNA-induced silencing complex). See, *e.g.*, Ketting *et al. Genes Dev* 2001 Oct 15;15(20):2654-9. and Hammond *Science* 2001 Aug 10;293(5532):1146-50.

dsRNA cleavage generally produces a plurality of iRNA species, each being a particular 21 to 23 nt fragment of a source dsRNA molecule. For example, iRNAs that include sequences complementary to overlapping regions and adjacent regions of a source dsRNA molecule may be present.

Regardless of the method of synthesis, the iRNA preparation can be prepared in a solution (*e.g.*, an aqueous and/or organic solution) that is appropriate for formulation. For example, the iRNA preparation can be precipitated and redissolved in pure double-distilled water, and lyophilized. The dried iRNA can then be resuspended in a solution appropriate for the intended formulation process.

Synthesis of modified and nucleotide surrogate iRNA agents is discussed below.

FORMULATION

The iRNA agents described herein can be formulated for administration to a subject

For ease of exposition the formulations, compositions and methods in this section are discussed largely with regard to unmodified iRNA agents. It should be understood, however,
5   that these formulations, compositions and methods can be practiced with other iRNA agents, e.g., modified iRNA agents, and such practice is within the invention.

A formulated iRNA composition can assume a variety of states. In some examples, the composition is at least partially crystalline, uniformly crystalline, and/or anhydrous (e.g., less than 80, 50, 30, 20, or 10% water). In another example, the iRNA is in an aqueous phase, e.g., in
10   a solution that includes water.

The aqueous phase or the crystalline compositions can, e.g., be incorporated into a delivery vehicle, e.g., a liposome (particularly for the aqueous phase) or a particle (e.g., a microparticle as can be appropriate for a crystalline composition). Generally, the iRNA composition is formulated in a manner that is compatible with the intended method of
5   administration (see, below).

In particular embodiments, the composition is prepared by at least one of the following methods: spray drying, lyophilization, vacuum drying, evaporation, fluid bed drying, or a combination of these techniques; or sonication with a lipid, freeze-drying, condensation and other self-assembly.

A iRNA preparation can be formulated in combination with another agent, e.g., another therapeutic agent or an agent that stabilizes a iRNA, e.g., a protein that complexes with iRNA to form an iRNP. Still other agents include chelators, e.g., EDTA (e.g., to remove divalent cations such as Mg^{2+}), salts, RNAse inhibitors (e.g., a broad specificity RNAse inhibitor such as RNAsin) and so forth.

In one embodiment, the iRNA preparation includes another iRNA agent, e.g., a second iRNA that can mediated RNAi with respect to a second gene, or with respect to the same gene. Still other preparation can include at least 3, 5, ten, twenty, fifty, or a hundred or more different iRNA species. Such iRNAs can mediated RNAi with respect to a similar number of different genes.

In one embodiment, the iRNA preparation includes at least a second therapeutic agent (*e.g.*, an agent other than an RNA or a DNA). For example, a iRNA composition for the treatment of a viral disease, *e.g.* HIV, might include a known antiviral agent (*e.g.*, a protease inhibitor or reverse transcriptase inhibitor). In another example, a iRNA composition for the treatment of a cancer might further comprise a chemotherapeutic agent.

Exemplary formulations are discussed below:

Liposomes

For ease of exposition the formulations, compositions and methods in this section are discussed largely with regard to unmodified iRNA agents. It should be understood, however, that these formulations, compositions and methods can be practiced with other iRNA agents, *e.g.*, modified iRNA s agents, and such practice is within the invention. An iRNA agent, *e.g.*, a double-stranded iRNA agent, or sRNA agent, (*e.g.*, a precursor, *e.g.*, a larger iRNA agent which can be processed into a sRNA agent, or a DNA which encodes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or sRNA agent, or precursor thereof) preparation can be formulated for delivery in a membranous molecular assembly, *e.g.*, a liposome or a micelle. As used herein, the term "liposome" refers to a vesicle composed of amphiphilic lipids arranged in at least one bilayer, *e.g.*, one bilayer or a plurality of bilayers. Liposomes include unilamellar and multilamellar vesicles that have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion contains the iRNA composition. The lipophilic material isolates the aqueous interior from an aqueous exterior, which typically does not include the iRNA composition, although in some examples, it may. Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological membranes, when liposomes are applied to a tissue, the liposomal bilayer fuses with bilayer of the cellular membranes. As the merging of the liposome and cell progresses, the internal aqueous contents that include the iRNA are delivered into the cell where the iRNA can specifically bind to a target RNA and can mediate RNAi. In some cases the liposomes are also specifically targeted, *e.g.*, to direct the iRNA to particular cell types, *e.g.*, to cells of the kidney, such as those described herein.

A liposome containing a iRNA can be prepared by a variety of methods.

In one example, the lipid component of a liposome is dissolved in a detergent so that micelles are formed with the lipid component. For example, the lipid component can be an

amphipathic cationic lipid or lipid conjugate. The detergent can have a high critical micelle concentration and may be nonionic. Exemplary detergents include cholate, CHAPS, octylglucoside, deoxycholate, and lauroyl sarcosine. The iRNA preparation is then added to the micelles that include the lipid component. The cationic groups on the lipid interact with the iRNA and condense around the iRNA to form a liposome. After condensation, the detergent is removed, e.g., by dialysis, to yield a liposomal preparation of iRNA.

If necessary a carrier compound that assists in condensation can be added during the condensation reaction, e.g., by controlled addition. For example, the carrier compound can be a polymer other than a nucleic acid (e.g., spermine or spermidine). pH can also be adjusted to favor condensation.

Further description of methods for producing stable polynucleotide delivery vehicles, which incorporate a polynucleotide/cationic lipid complex as structural components of the delivery vehicle, are described in, e.g., WO 96/37194. Liposome formation can also include one or more aspects of exemplary methods described in Felgner, P. L. et al., Proc. Natl. Acad. Sci., USA 8:7413-7417, 1987; U.S. Pat. No. 4,897,355; U.S. Pat. No. 5,171,678; Bangham, et al. M. Mol. Biol. 23:238, 1965; Olson, et al. Biochim. Biophys. Acta 557:9, 1979; Szoka, et al. Proc. Natl. Acad. Sci. 75: 4194, 1978; Mayhew, et al. Biochim. Biophys. Acta 775:169, 1984; Kim, et al. Biochim. Biophys. Acta 728:339, 1983; and Fukunaga, et al. Endocrinol. 115:757, 1984. Commonly used techniques for preparing lipid aggregates of appropriate size for use as delivery vehicles include sonication and freeze-thaw plus extrusion (see, e.g., Mayer, et al. Biochim. Biophys. Acta 858:161, 1986). Microfluidization can be used when consistently small (50 to 200 nm) and relatively uniform aggregates are desired (Mayhew, et al. Biochim. Biophys. Acta 775:169, 1984). These methods are readily adapted to packaging iRNA preparations into liposomes.

Liposomes that are pH-sensitive or negatively-charged, entrap nucleic acid molecules rather than complex with them. Since both the nucleic acid molecules and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some nucleic acid molecules are entrapped within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver DNA encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou et al., Journal of Controlled Release, 19, (1992) 269-274).

One major type of liposomal composition includes phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

Examples of other methods to introduce liposomes into cells *in vitro* and *in vivo* include U.S. Pat. No. 5,283,185; U.S. Pat. No. 5,171,678; WO 94/00569; WO 93/24640; WO 91/16024; Felgner, *J. Biol. Chem.* 269:2550, 1994; Nabel, *Proc. Natl. Acad. Sci.* 90:11307, 1993; Nabel, *Human Gene Ther.* 3:649, 1992; Gershon, *Biochem.* 32:7143, 1993; and Strauss *EMBO J.* 11:417, 1992.

In one embodiment, cationic liposomes are used. Cationic liposomes possess the advantage of being able to fuse to the cell membrane. Non-cationic liposomes, although not able to fuse as efficiently with the plasma membrane, are taken up by macrophages *in vivo* and can be used to deliver iRNAs to macrophages.

Further advantages of liposomes include: liposomes obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of water and lipid soluble drugs; liposomes can protect encapsulated iRNAs in their internal compartments from metabolism and degradation (Rosoff, in "Pharmaceutical Dosage Forms," Lieberman, Rieger and Banker (Eds.), 1988, volume 1, p. 245). Important considerations in the preparation of liposome formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

A positively charged synthetic cationic lipid, N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) can be used to form small liposomes that interact spontaneously with nucleic acid to form lipid-nucleic acid complexes which are capable of fusing with the negatively charged lipids of the cell membranes of tissue culture cells, resulting in delivery of iRNA (see, *e.g.*, Felgner, P. L. *et al.*, Proc. Natl. Acad. Sci., USA 8:7413-7417, 1987 and U.S. Pat. No. 4,897,355 for a description of DOTMA and its use with DNA).

A DOTMA analogue, 1,2-bis(oleoyloxy)-3-(trimethylammonia)propane (DOTAP) can be used in combination with a phospholipid to form DNA-complexing vesicles. Lipofectin™

Bethesda Research Laboratories, Gaithersburg, Md.) is an effective agent for the delivery of highly anionic nucleic acids into living tissue culture cells that comprise positively charged DOTMA liposomes which interact spontaneously with negatively charged polynucleotides to form complexes. When enough positively charged liposomes are used, the net charge on the resulting complexes is also positive. Positively charged complexes prepared in this way spontaneously attach to negatively charged cell surfaces, fuse with the plasma membrane, and efficiently deliver functional nucleic acids into, for example, tissue culture cells. Another commercially available cationic lipid, 1,2-bis(oleoyloxy)-3,3-(trimethylammonia)propane ("DOTAP") (Boehringer Mannheim, Indianapolis, Indiana) differs from DOTMA in that the oleoyl moieties are linked by ester, rather than ether linkages.

Other reported cationic lipid compounds include those that have been conjugated to a variety of moieties including, for example, carboxyspermine which has been conjugated to one of two types of lipids and includes compounds such as 5-carboxyspermylglycine dioctaoleoylamide ("DOGS") (Transfectam™, Promega, Madison, Wisconsin) and dipalmitoylphosphatidylethanolamine 5-carboxyspermyl-amide ("DPPES") (see, *e.g.*, U.S. Pat. No. 5,171,678).

Another cationic lipid conjugate includes derivatization of the lipid with cholesterol ("DC-Chol") which has been formulated into liposomes in combination with DOPE (See, Gao, X. and Huang, L., Biochim. Biophys. Res. Commun. 179:280, 1991). Lipopolylysine, made by conjugating polylysine to DOPE, has been reported to be effective for transfection in the presence of serum (Zhou, X. *et al.*, Biochim. Biophys. Acta 1065:8, 1991). For certain cell lines, these liposomes containing conjugated cationic lipids, are said to exhibit lower toxicity and provide more efficient transfection than the DOTMA-containing compositions. Other commercially available cationic lipid products include DMRIE and DMRIE-HP (Vical, La Jolla, California) and Lipofectamine (DOSPA) (Life Technology, Inc., Gaithersburg, Maryland). Other cationic lipids suitable for the delivery of oligonucleotides are described in WO 98/39359 and WO 96/37194.

Liposomal formulations are particularly suited for topical administration, liposomes present several advantages over other formulations. Such advantages include reduced side effects related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer iRNA, into the skin. In some implementations, liposomes are used for delivering iRNA to epidermal cells and also to

enhance the penetration of iRNA into dermal tissues, *e.g.*, into skin. For example, the liposomes can be applied topically. Topical delivery of drugs formulated as liposomes to the skin has been documented (see, *e.g.*, Weiner *et al.*, *Journal of Drug Targeting*, 1992, vol. 2,405-410 and du Plessis *et al.*, *Antiviral Research*, 18, 1992, 259-265; Mannino, R. J. and Fould-Fogerite, S., Biotechniques 6:682-690, 1988; Itani, T. *et al.* Gene 56:267-276. 1987; Nicolau, C. *et al.* Meth. Enz. 149:157-176, 1987; Straubinger, R. M. and Papahadjopoulos, D. Meth. Enz. 101:512-527, 1983; Wang, C. Y. and Huang, L., Proc. Natl. Acad. Sci. USA 84:7851-7855, 1987).

Non-ionic liposomal systems have also been examined to determine their utility in the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant and cholesterol. Non-ionic liposomal formulations comprising Novasome I (glyceryl dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and Novasome II (glyceryl distearate/cholesterol/polyoxyethylene-10-stearyl ether) were used to deliver a drug into the dermis of mouse skin. Such formulations with iRNA are useful for treating a dermatological disorder.

Liposomes that include iRNA can be made highly deformable. Such deformability can enable the liposomes to penetrate through pore that are smaller than the average radius of the liposome. For example, transfersomes are a type of deformable liposomes. Transferosomes can be made by adding surface edge activators, usually surfactants, to a standard liposomal composition. Transfersomes that include iRNA can be delivered, for example, subcutaneously by infection in order to deliver iRNA to keratinocytes in the skin. In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. In addition, due to the lipid properties, these transferosomes can be self-optimizing (adaptive to the shape of pores, *e.g.*, in the skin), self-repairing, and can frequently reach their targets without fragmenting, and often self-loading. The iRNA agents can include an RRMS tethered to a moiety which improves association with a liposome.

Surfactants

For ease of exposition the formulations, compositions and methods in this section are discussed largely with regard to unmodified iRNA agents. It should be understood, however, that these formulations, compositions and methods can be practiced with other iRNA agents, *e.g.*, modified iRNA agents, and such practice is within the invention. Surfactants find wide application in formulations such as emulsions (including microemulsions) and liposomes (see above). iRNA (or a precursor, *e.g.*, a larger dsRNA which can be processed into a iRNA, or a

DNA which encodes a iRNA or precursor) compositions can include a surfactant. In one embodiment, the iRNA is formulated as an emulsion that includes a surfactant. The most common way of classifying and ranking the properties of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group provides the most useful means for categorizing the different surfactants used in formulations (Rieger, in "Pharmaceutical Dosage Forms," Marcel Dekker, Inc., New York, NY, 1988, p. 285).

If the surfactant molecule is not ionized, it is classified as a nonionic surfactant. Nonionic surfactants find wide application in pharmaceutical products and are usable over a wide range of pH values. In general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic esters such as ethylene glycol esters, propylene glycol esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant is classified as anionic. Anionic surfactants include carboxylates such as soaps, acyl lactylates, acyl amides of amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and sulfosuccinates, and phosphates. The most important members of the anionic surfactant class are the alkyl sulfates and the soaps.

If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant is classified as cationic. Cationic surfactants include quaternary ammonium salts and ethoxylated amines. The quaternary ammonium salts are the most used members of this class.

If the surfactant molecule has the ability to carry either a positive or negative charge, the surfactant is classified as amphoteric. Amphoteric surfactants include acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, in "Pharmaceutical Dosage Forms," Marcel Dekker, Inc., New York, NY, 1988, p. 285).

Micelles and other Membranous Formulations

For ease of exposition the micelles and other formulations, compositions and methods in this section are discussed largely with regard to unmodified iRNA agents. It should be understood, however, that these micelles and other formulations, compositions and methods can be practiced with other iRNA agents, *e.g.*, modified iRNA agents, and such practice is within the invention. The iRNA agent, *e.g.*, a double-stranded iRNA agent, or sRNA agent, (*e.g.*, a precursor, *e.g.*, a larger iRNA agent which can be processed into a sRNA agent, or a DNA which encodes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or sRNA agent, or precursor thereof)) composition can be provided as a micellar formulation. "Micelles" are defined herein as a particular type of molecular assembly in which amphipathic molecules are arranged in a spherical structure such that all the hydrophobic portions of the molecules are directed inward, leaving the hydrophilic portions in contact with the surrounding aqueous phase. The converse arrangement exists if the environment is hydrophobic.

A mixed micellar formulation suitable for delivery through transdermal membranes may be prepared by mixing an aqueous solution of the iRNA composition, an alkali metal C_8 to C_{22} alkyl sulphate, and a micelle forming compounds. Exemplary micelle forming compounds include lecithin, hyaluronic acid, pharmaceutically acceptable salts of hyaluronic acid, glycolic acid, lactic acid, chamomile extract, cucumber extract, oleic acid, linoleic acid, linolenic acid, monoolein, monooleates, monolaurates, borage oil, evening of primrose oil, menthol, trihydroxy oxo cholanyl glycine and pharmaceutically acceptable salts thereof, glycerin, polyglycerin, lysine, polylysine, triolein, polyoxyethylene ethers and analogues thereof, polidocanol alkyl ethers and analogues thereof, chenodeoxycholate, deoxycholate, and mixtures thereof. The micelle forming compounds may be added at the same time or after addition of the alkali metal alkyl sulphate. Mixed micelles will form with substantially any kind of mixing of the ingredients but vigorous mixing is preferred in order to provide smaller size micelles.

In one method a first micellar composition is prepared which contains the iRNA composition and at least the alkali metal alkyl sulphate. The first micellar composition is then mixed with at least three micelle forming compounds to form a mixed micellar composition. In another method, the micellar composition is prepared by mixing the iRNA composition, the alkali metal alkyl sulphate and at least one of the micelle forming compounds, followed by addition of the remaining micelle forming compounds, with vigorous mixing.

Phenol and/or m-cresol may be added to the mixed micellar composition to stabilize the formulation and protect against bacterial growth. Alternatively, phenol and/or m-cresol may be added with the micelle forming ingredients. An isotonic agent such as glycerin may also be added after formation of the mixed micellar composition.

For delivery of the micellar formulation as a spray, the formulation can be put into an aerosol dispenser and the dispenser is charged with a propellant. The propellant, which is under pressure, is in liquid form in the dispenser. The ratios of the ingredients are adjusted so that the aqueous and propellant phases become one, *i.e.* there is one phase. If there are two phases, it is necessary to shake the dispenser prior to dispensing a portion of the contents, *e.g.* through a metered valve. The dispensed dose of pharmaceutical agent is propelled from the metered valve in a fine spray.

The preferred propellants are hydrogen-containing chlorofluorocarbons, hydrogen-containing fluorocarbons, dimethyl ether and diethyl ether. Even more preferred is HFA 134a (1,1,1,2 tetrafluoroethane).

The specific concentrations of the essential ingredients can be determined by relatively straightforward experimentation. For absorption through the oral cavities, it is often desirable to increase, *e.g.* at least double or triple, the dosage for through injection or administration through the gastrointestinal tract.

The iRNA agents can include an RRMS tethered to a moiety which improves association with a micelle or other membranous formulation.

Particles

For ease of exposition the particles, formulations, compositions and methods in this section are discussed largely with regard to unmodified iRNA agents. It should be understood, however, that these particles, formulations, compositions and methods can be practiced with other iRNA agents, *e.g.*, modified iRNA agents, and such practice is within the invention. In another embodiment, an iRNA agent, *e.g.*, a double-stranded iRNA agent, or sRNA agent, (*e.g.*, a precursor, *e.g.*, a larger iRNA agent which can be processed into a sRNA agent, or a DNA which encodes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or sRNA agent, or precursor thereof) preparations may be incorporated into a particle, *e.g.*, a microparticle. Microparticles can be produced by spray-drying, but may also be produced by other methods including

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lyophilization, evaporation, fluid bed drying, vacuum drying, or a combination of these techniques. See below for further description.

Sustained-Release Formulations. An iRNA agent, *e.g.*, a double-stranded iRNA agent, or sRNA agent, (*e.g.*, a precursor, *e.g.*, a larger iRNA agent which can be processed into a sRNA agent, or a DNA which encodes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or sRNA agent, or precursor thereof) described herein can be formulated for controlled, *e.g.*, slow release. Controlled release can be achieved by disposing the iRNA within a structure or substance which impedes its release. *E.g.*, iRNA can be disposed within a porous matrix or in an erodable matrix, either of which allow release of the iRNA over a period of time.

Polymeric particles, *e.g.*, polymeric in microparticles can be used as a sustained-release reservoir of iRNA that is taken up by cells only released from the microparticle through biodegradation. The polymeric particles in this embodiment should therefore be large enough to preclude phagocytosis (*e.g.*, larger than 10 μm and preferably larger than 20 μm). Such particles can be produced by the same methods to make smaller particles, but with less vigorous mixing of the first and second emulsions. That is to say, a lower homogenization speed, vortex mixing speed, or sonication setting can be used to obtain particles having a diameter around 100 μm rather than 10 μm. The time of mixing also can be altered.

Larger microparticles can be formulated as a suspension, a powder, or an implantable solid, to be delivered by intramuscular, subcutaneous, intradermal, intravenous, or intraperitoneal injection; via inhalation (intranasal or intrapulmonary); orally; or by implantation. These particles are useful for delivery of any iRNA when slow release over a relatively long term is desired. The rate of degradation, and consequently of release, varies with the polymeric formulation.

Microparticles preferably include pores, voids, hollows, defects or other interstitial spaces that allow the fluid suspension medium to freely permeate or perfuse the particulate boundary. For example, the perforated microstructures can be used to form hollow, porous spray dried microspheres.

Polymeric particles containing iRNA (*e.g.*, a sRNA) can be made using a double emulsion technique, for instance. First, the polymer is dissolved in an organic solvent. A preferred polymer is polylactic-co-glycolic acid (PLGA), with a lactic/glycolic acid weight ratio of 65:35, 50:50, or 75:25. Next, a sample of nucleic acid suspended in aqueous solution is added

to the polymer solution and the two solutions are mixed to form a first emulsion. The solutions can be mixed by vortexing or shaking, and in a preferred method, the mixture can be sonicated. Most preferable is any method by which the nucleic acid receives the least amount of damage in the form of nicking, shearing, or degradation, while still allowing the formation of an appropriate emulsion. For example, acceptable results can be obtained with a Vibra-cell model VC-250 sonicator with a 1/8" microtip probe, at setting #3.

Spray-Drying. An iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, (e.g., a precursor, e.g., a larger iRNA agent which can be processed into a sRNA agent, or a DNA which encodes an iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, or precursor thereof)) can be prepared by spray drying. Spray dried iRNA can be administered to a subject or be subjected to further formulation. A pharmaceutical composition of iRNA can be prepared by spray drying a homogeneous aqueous mixture that includes a iRNA under conditions sufficient to provide a dispersible powdered composition, e.g., a pharmaceutical composition. The material for spray drying can also include one or more of: a pharmaceutically acceptable excipient, or a dispersibility-enhancing amount of a physiologically acceptable, water-soluble protein. The spray-dried product can be a dispersible powder that includes the iRNA.

Spray drying is a process that converts a liquid or slurry material to a dried particulate form. Spray drying can be used to provide powdered material for various administrative routes including inhalation. See, for example, M. Sacchetti and M. M. Van Oort in: Inhalation Aerosols: Physical and Biological Basis for Therapy, A. J. Hickey, ed. Marcel Dekkar, New York, 1996.

Spray drying can include atomizing a solution, emulsion, or suspension to form a fine mist of droplets and drying the droplets. The mist can be projected into a drying chamber (e.g., a vessel, tank, tubing, or coil) where it contacts a drying gas. The mist can include solid or liquid pore forming agents. The solvent and pore forming agents evaporate from the droplets into the drying gas to solidify the droplets, simultaneously forming pores throughout the solid. The solid (typically in a powder, particulate form) then is separated from the drying gas and collected.

Spray drying includes bringing together a highly dispersed liquid, and a sufficient volume of air (e.g., hot air) to produce evaporation and drying of the liquid droplets. The preparation to be spray dried can be any solution, course suspension, slurry, colloidal dispersion, or paste that may be atomized using the selected spray drying apparatus. Typically, the feed is sprayed into a current of warm filtered air that evaporates the solvent and conveys the dried product to a collector. The spent air is then exhausted with the solvent. Several different types of apparatus

may be used to provide the desired product. For example, commercial spray dryers manufactured by Buchi Ltd. or Niro Corp. can effectively produce particles of desired size.

Spray-dried powdered particles can be approximately spherical in shape, nearly uniform in size and frequently hollow. There may be some degree of irregularity in shape depending upon the incorporated medicament and the spray drying conditions. In many instances the dispersion stability of spray-dried microspheres appears to be more effective if an inflating agent (or blowing agent) is used in their production. Particularly preferred embodiments may comprise an emulsion with an inflating agent as the disperse or continuous phase (the other phase being aqueous in nature). An inflating agent is preferably dispersed with a surfactant solution, using, for instance, a commercially available microfluidizer at a pressure of about 5000 to 15,000 psi. This process forms an emulsion, preferably stabilized by an incorporated surfactant, typically comprising submicron droplets of water immiscible blowing agent dispersed in an aqueous continuous phase. The formation of such dispersions using this and other techniques are common and well known to those in the art. The blowing agent is preferably a fluorinated compound (e.g. perfluorohexane, perfluorooctyl bromide, perfluorodecalin, perfluorobutyl ethane) which vaporizes during the spray-drying process, leaving behind generally hollow, porous aerodynamically light microspheres. As will be discussed in more detail below, other suitable blowing agents include chloroform, freons, and hydrocarbons. Nitrogen gas and carbon dioxide are also contemplated as a suitable blowing agent.

Although the perforated microstructures are preferably formed using a blowing agent as described above, it will be appreciated that, in some instances, no blowing agent is required and an aqueous dispersion of the medicament and surfactant(s) are spray dried directly. In such cases, the formulation may be amenable to process conditions (e.g., elevated temperatures) that generally lead to the formation of hollow, relatively porous microparticles. Moreover, the medicament may possess special physicochemical properties (e.g., high crystallinity, elevated melting temperature, surface activity, etc.) that make it particularly suitable for use in such techniques.

The perforated microstructures may optionally be associated with, or comprise, one or more surfactants. Moreover, miscible surfactants may optionally be combined with the suspension medium liquid phase. It will be appreciated by those skilled in the art that the use of surfactants may further increase dispersion stability, simplify formulation procedures or increase bioavailability upon administration. Of course combinations of surfactants, including the use of

one or more in the liquid phase and one or more associated with the perforated microstructures are contemplated as being within the scope of the invention. By "associated with or comprise" it is meant that the structural matrix or perforated microstructure may incorporate, adsorb, absorb, be coated with or be formed by the surfactant.

5   Surfactants suitable for use include any compound or composition that aids in the formation and maintenance of the stabilized respiratory dispersions by forming a layer at the interface between the structural matrix and the suspension medium. The surfactant may comprise a single compound or any combination of compounds, such as in the case of co-surfactants. Particularly preferred surfactants are substantially insoluble in the propellant, nonfluorinated, and

10   selected from the group consisting of saturated and unsaturated lipids, nonionic detergents, nonionic block copolymers, ionic surfactants, and combinations of such agents. It should be emphasized that, in addition to the aforementioned surfactants, suitable (i.e. biocompatible) fluorinated surfactants are compatible with the teachings herein and may be used to provide the desired stabilized preparations.

5   Lipids, including phospholipids, from both natural and synthetic sources may be used in varying concentrations to form a structural matrix. Generally, compatible lipids comprise those that have a gel to liquid crystal phase transition greater than about 40° C. Preferably, the incorporated lipids are relatively long chain (i.e. C_6 -C_{22}) saturated lipids and more preferably comprise phospholipids. Exemplary phospholipids useful in the disclosed stabilized preparations comprise egg phosphatidylcholine, dilauroylphosphatidylcholine, dioleylphosphatidylcholine, dipalmitoylphosphatidyl-choline, disteroylphosphatidylcholine, short-chain phosphatidylcholines, phosphatidylethanolamine, dioleylphosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, phosphatidylinositol, glycolipids, ganglioside GM1, sphingomyelin, phosphatidic acid, cardiolipin; lipids bearing polymer chains such as, polyethylene glycol, chitin, hyaluronic acid, or polyvinylpyrrolidone; lipids bearing sulfonated mono-, di-, and polysaccharides; fatty acids such as palmitic acid, stearic acid, and oleic acid; cholesterol, cholesterol esters, and cholesterol hemisuccinate. Due to their excellent biocompatibility characteristics, phospholipids and combinations of phospholipids and poloxamers are particularly suitable for use in the stabilized dispersions disclosed herein.

Compatible nonionic detergents comprise: sorbitan esters including sorbitan trioleate (Spans™ 85), sorbitan sesquioleate, sorbitan monooleate, sorbitan monolaurate, polyoxyethylene (20) sorbitan monolaurate, and polyoxyethylene (20) sorbitan monooleate, oleyl polyoxyethylene

(2) ether, stearyl polyoxyethylene (2) ether, lauryl polyoxyethylene (4) ether, glycerol esters, and sucrose esters. Other suitable nonionic detergents can be easily identified using McCutcheon's Emulsifiers and Detergents (McPublishing Co., Glen Rock, N.J.). Preferred block copolymers include diblock and triblock copolymers of polyoxyethylene and polyoxypropylene, including poloxamer 188 (Pluronic.RTM. F68), poloxamer 407 (Pluronic.RTM. F-127), and poloxamer 338. Ionic surfactants such as sodium sulfosuccinate, and fatty acid soaps may also be utilized. In preferred embodiments, the microstructures may comprise oleic acid or its alkali salt.

In addition to the aforementioned surfactants, cationic surfactants or lipids are preferred especially in the case of delivery of an iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, (e.g., a precursor, e.g., a larger iRNA agent which can be processed into a sRNA agent, or a DNA which encodes an iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, or precursor thereof). Examples of suitable cationic lipids include: DOTMA, N-[-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium-chloride; DOTAP,1,2-dioleyloxy-3-(trimethylammonio)propane; and DOTB, 1,2-dioleyl-3-(4'-trimethylammonio)butanoyl-sn-glycerol. Polycationic amino acids such as polylysine, and polyarginine are also contemplated.

For the spraying process, such spraying methods as rotary atomization, pressure atomization and two-fluid atomization can be used. Examples of the devices used in these processes include "Parubisu [phonetic rendering] Mini-Spray GA-32" and "Parubisu Spray Drier DL-41", manufactured by Yamato Chemical Co., or "Spray Drier CL-8," "Spray Drier L-8," "Spray Drier FL-12," "Spray Drier FL-16" or "Spray Drier FL-20," manufactured by Okawara Kakoki Co., can be used for the method of spraying using rotary-disk atomizer.

While no particular restrictions are placed on the gas used to dry the sprayed material, it is recommended to use air, nitrogen gas or an inert gas. The temperature of the inlet of the gas used to dry the sprayed materials such that it does not cause heat deactivation of the sprayed material. The range of temperatures may vary between about 50ºC to about 200ºC, preferably between about 50ºC and 100ºC. The temperature of the outlet gas used to dry the sprayed material, may vary between about 0ºC and about 150ºC, preferably between 0ºC and 90ºC, and even more preferably between 0ºC and 60ºC.

The spray drying is done under conditions that result in substantially amorphous powder of homogeneous constitution having a particle size that is respirable, a low moisture content and flow characteristics that allow for ready aerosolization. Preferably the particle size of the resulting powder is such that more than about 98% of the mass is in particles having a diameter

of about 10 μm or less with about 90% of the mass being in particles having a diameter less than 5 μm. Alternatively, about 95% of the mass will have particles with a diameter of less than 10 μm with about 80% of the mass of the particles having a diameter of less than 5 μm.

The dispersible pharmaceutical-based dry powders that include the iRNA preparation may optionally be combined with pharmaceutical carriers or excipients which are suitable for respiratory and pulmonary administration. Such carriers may serve simply as bulking agents when it is desired to reduce the iRNA concentration in the powder which is being delivered to a patient, but may also serve to enhance the stability of the iRNA compositions and to improve the dispersibility of the powder within a powder dispersion device in order to provide more efficient and reproducible delivery of the iRNA and to improve handling characteristics of the iRNA such as flowability and consistency to facilitate manufacturing and powder filling.

Such carrier materials may be combined with the drug prior to spray drying, *i.e.*, by adding the carrier material to the purified bulk solution. In that way, the carrier particles will be formed simultaneously with the drug particles to produce a homogeneous powder. Alternatively, the carriers may be separately prepared in a dry powder form and combined with the dry powder drug by blending. The powder carriers will usually be crystalline (to avoid water absorption), but might in some cases be amorphous or mixtures of crystalline and amorphous. The size of the carrier particles may be selected to improve the flowability of the drug powder, typically being in the range from 25 μm to 100 μm. A preferred carrier material is crystalline lactose having a size in the above-stated range.

Powders prepared by any of the above methods will be collected from the spray dryer in a conventional manner for subsequent use. For use as pharmaceuticals and other purposes, it will frequently be desirable to disrupt any agglomerates which may have formed by screening or other conventional techniques. For pharmaceutical uses, the dry powder formulations will usually be measured into a single dose, and the single dose sealed into a package. Such packages are particularly useful for dispersion in dry powder inhalers, as described in detail below. Alternatively, the powders may be packaged in multiple-dose containers.

Methods for spray drying hydrophobic and other drugs and components are described in U.S. Pat. Nos. 5,000,888; 5,026,550; 4,670,419, 4,540,602; and 4,486,435. Bloch and Speison (1983) Pharm. Acta Helv 58:14-22 teaches spray drying of hydrochlorothiazide and chlorthalidone (lipophilic drugs) and a hydrophilic adjuvant (pentaerythritol) in azeotropic solvents of dioxane-water and 2-ethoxyethanol-water. A number of Japanese Patent application

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Abstracts relate to spray drying of hydrophilic-hydrophobic product combinations, including JP 806766; JP 7242568; JP 7101884; JP 7101883; JP 71018982; JP 7101881; and JP 4036233. Other foreign patent publications relevant to spray drying hydrophilic-hydrophobic product combinations include FR 2594693; DE 2209477; and WO 88/07870.

5   LYOPHILIZATION.

An iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, (e.g., a precursor, e.g., a larger iRNA agent which can be processed into a sRNA agent, or a DNA which encodes an iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, or precursor thereof) preparation can be made by lyophilization. Lyophilization is a freeze-drying process in which
10   water is sublimed from the composition after it is frozen. The particular advantage associated with the lyophilization process is that biologicals and pharmaceuticals that are relatively unstable in an aqueous solution can be dried without elevated temperatures (thereby eliminating the adverse thermal effects), and then stored in a dry state where there are few stability problems. With respect to the instant invention such techniques are particularly compatible with the
15   incorporation of nucleic acids in perforated microstructures without compromising physiological activity. Methods for providing lyophilized particulates are known to those of skill in the art and it would clearly not require undue experimentation to provide dispersion compatible microstructures in accordance with the teachings herein. Accordingly, to the extent that lyophilization processes may be used to provide microstructures having the desired porosity and
20   size, they are conformance with the teachings herein and are expressly contemplated as being within the scope of the instant invention.

Targeting

For ease of exposition the formulations, compositions and methods in this section are discussed largely with regard to unmodified iRNAs. It should be understood, however, that
5   these formulations, compositions and methods can be practiced with other iRNA agents, e.g., modified iRNA agents, and such practice is within the invention.

In some embodiments, an iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, (e.g., a precursor, e.g., a larger iRNA agent which can be processed into a sRNA agent, or a DNA which encodes an iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, or
0   precursor thereof) is targeted to a particular cell. For example, a liposome or particle or other structure that includes a iRNA can also include a targeting moiety that recognizes a specific

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molecule on a target cell. The targeting moiety can be a molecule with a specific affinity for a target cell. Targeting moieties can include antibodies directed against a protein found on the surface of a target cell, or the ligand or a receptor-binding portion of a ligand for a molecule found on the surface of a target cell. For example, the targeting moiety can recognize a cancer-specific antigen of the kidney (e.g., G250, CA15-3, CA19-9, CEA, or HER2/neu) or a viral antigen, thus delivering the iRNA to a cancer cell or a virus-infected cell. Exemplary targeting moieties include antibodies (such as IgM, IgG, IgA, IgD, and the like, or a functional portions thereof), ligands for cell surface receptors (e.g., ectodomains thereof).

Table 6 provides a number of antigens which can be used to target an iRNA to a selected cell, such as when targeting of the iRNA agent to a tissue other than the kidney is desired.

<u>Table 6</u>. Targeting Antigens

ANTIGEN	Exemplary tumor tissue
CEA (carcinoembryonic antigen)	colon, breast, lung
PSA (prostate specific antigen)	prostate cancer
CA-125	ovarian cancer
CA 15-3	breast cancer
CA 19-9	breast cancer
HER2/neu	breast cancer
α-feto protein	testicular cancer, hepatic cancer
β-HCG (human chorionic gonadotropin)	testicular cancer, choriocarcinoma
MUC-1	breast cancer
Estrogen receptor	breast cancer, uterine cancer
Progesterone receptor	breast cancer, uterine cancer
EGFr (epidermal growth factor receptor)	bladder cancer

In one embodiment, the targeting moiety is attached to a liposome. For example, US Patent 6,245,427 describes a method for targeting a liposome using a protein or peptide. In

another example, a cationic lipid component of the liposome is derivatized with a targeting moiety. For example, WO 96/37194 describes converting N-glutaryldioleoylphosphatidyl ethanolamine to a N-hydroxysuccinimide activated ester. The product was then coupled to an RGD peptide.

GENES AND DISEASES

In one aspect, the invention features, a method of treating a subject at risk for or afflicted with unwanted cell proliferation, *e.g.*, malignant or nonmalignant cell proliferation. The method includes:

providing an iRNA agent, *e.g.*, an sRNA or iRNA agent described herein, *e.g.*, an iRNA having a structure described herein, where the iRNA is homologous to and can silence, *e.g.*, by cleavage, a gene which promotes unwanted cell proliferation;

administering an iRNA agent, *e.g.*, an sRNA or iRNA agent described herein to a subject, preferably a human subject,

thereby treating the subject.

In a preferred embodiment the gene is a growth factor or growth factor receptor gene, a kinase, *e.g.*, a protein tyrosine, serine or threonine kinase gene, an adaptor protein gene, a gene encoding a G protein superfamily molecule, or a gene encoding a transcription factor.

In a preferred embodiment the iRNA agent silences the PDGF beta gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted PDGF beta expression, *e.g.*, testicular and lung cancers.

In another preferred embodiment the iRNA agent silences the Erb-B gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted Erb-B expression, *e.g.*, breast cancer.

In a preferred embodiment the iRNA agent silences the Src gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted Src expression, *e.g.*, colon cancers.

In a preferred embodiment the iRNA agent silences the CRK gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted CRK expression, *e.g.*, colon and lung cancers.

In a preferred embodiment the iRNA agent silences the GRB2 gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted GRB2 expression, *e.g.*, squamous cell carcinoma.

In another preferred embodiment the iRNA agent silences the RAS gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted RAS expression, *e.g.*, pancreatic, colon and lung cancers, and chronic leukemia.

In another preferred embodiment the iRNA agent silences the MEKK gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted MEKK expression, *e.g.*, squamous cell carcinoma, melanoma or leukemia.

In another preferred embodiment the iRNA agent silences the JNK gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted JNK expression, *e.g.*, pancreatic or breast cancers.

In a preferred embodiment the iRNA agent silences the RAF gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted RAF expression, *e.g.*, lung cancer or leukemia.

In a preferred embodiment the iRNA agent silences the Erk1/2 gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted Erk1/2 expression, *e.g.*, lung cancer.

In another preferred embodiment the iRNA agent silences the PCNA(p21) gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted PCNA expression, *e.g.*, lung cancer.

In a preferred embodiment the iRNA agent silences the MYB gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted MYB expression, *e.g.*, colon cancer or chronic myelogenous leukemia.

In a preferred embodiment the iRNA agent silences the c-MYC gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted c-MYC expression, e.g., Burkitt's lymphoma or neuroblastoma.

In another preferred embodiment the iRNA agent silences the JUN gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted JUN expression, e.g., ovarian, prostate or breast cancers.

In another preferred embodiment the iRNA agent silences the FOS gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted FOS expression, e.g., skin or prostate cancers.

In a preferred embodiment the iRNA agent silences the BCL-2 gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted BCL-2 expression, e.g., lung or prostate cancers or Non-Hodgkin lymphoma.

In a preferred embodiment the iRNA agent silences the Cyclin D gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted Cyclin D expression, e.g., esophageal and colon cancers.

In a preferred embodiment the iRNA agent silences the VEGF gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted VEGF expression, e.g., esophageal and colon cancers.

In a preferred embodiment the iRNA agent silences the EGFR gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted EGFR expression, e.g., breast cancer.

In another preferred embodiment the iRNA agent silences the Cyclin A gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted Cyclin A expression, e.g., lung and cervical cancers.

In another preferred embodiment the iRNA agent silences the Cyclin E gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted Cyclin E expression, e.g., lung and breast cancers.

In another preferred embodiment the iRNA agent silences the WNT-1 gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted WNT-1 expression, e.g., basal cell carcinoma.

In another preferred embodiment the iRNA agent silences the beta-catenin gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted beta-catenin expression, e.g., adenocarcinoma or hepatocellular carcinoma.

In another preferred embodiment the iRNA agent silences the c-MET gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted c-MET expression, e.g., hepatocellular carcinoma.

In another preferred embodiment the iRNA agent silences the PKC gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted PKC expression, e.g., breast cancer.

In a preferred embodiment the iRNA agent silences the NFKB gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted NFKB expression, e.g., breast cancer.

In a preferred embodiment the iRNA agent silences the STAT3 gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted STAT3 expression, e.g., prostate cancer.

In another preferred embodiment the iRNA agent silences the survivin gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted survivin expression, e.g., cervical or pancreatic cancers.

In another preferred embodiment the iRNA agent silences the Her2/Neu gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted Her2/Neu expression, e.g., breast cancer.

In another preferred embodiment the iRNA agent silences the topoisomerase I gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted topoisomerase I expression, e.g., ovarian and colon cancers.

In a preferred embodiment the iRNA agent silences the topoisomerase II alpha gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted topoisomerase II expression, *e.g.*, breast and colon cancers.

In a preferred embodiment the iRNA agent silences mutations in the p73 gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted p73 expression, *e.g.*, colorectal adenocarcinoma.

In a preferred embodiment the iRNA agent silences mutations in the p21(WAF1/CIP1) gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted p21(WAF1/CIP1) expression, *e.g.*, liver cancer.

In a preferred embodiment the iRNA agent silences mutations in the p27(KIP1) gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted p27(KIP1) expression, *e.g.*, liver cancer.

In a preferred embodiment the iRNA agent silences mutations in the PPM1D gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted PPM1D expression, *e.g.*, breast cancer.

In a preferred embodiment the iRNA agent silences mutations in the RAS gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted RAS expression, *e.g.*, breast cancer.

In another preferred embodiment the iRNA agent silences mutations in the caveolin I gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted caveolin I expression, *e.g.*, esophageal squamous cell carcinoma.

In another preferred embodiment the iRNA agent silences mutations in the MIB I gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted MIB I expression, *e.g.*, male breast carcinoma (MBC).

In another preferred embodiment the iRNA agent silences mutations in the MTAI gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted MTAI expression, *e.g.*, ovarian carcinoma.

In another preferred embodiment the iRNA agent silences mutations in the M68 gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted M68 expression, *e.g.*, human adenocarcinomas of the esophagus, stomach, colon, and rectum.

In preferred embodiments the iRNA agent silences mutations in tumor suppressor genes, and thus can be used as a method to promote apoptotic activity in combination with chemotherapeutics.

In a preferred embodiment the iRNA agent silences mutations in the p53 tumor suppressor gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted p53 expression, *e.g.*, gall bladder, pancreatic and lung cancers.

In a preferred embodiment the iRNA agent silences mutations in the p53 family member DN-p63, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted DN-p63 expression, *e.g.*, squamous cell carcinoma

In a preferred embodiment the iRNA agent silences mutations in the pRb tumor suppressor gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted pRb expression, *e.g.*, oral squamous cell carcinoma

In a preferred embodiment the iRNA agent silences mutations in the APC1 tumor suppressor gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted APC1 expression, *e.g.*, colon cancer.

In a preferred embodiment the iRNA agent silences mutations in the BRCA1 tumor suppressor gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted BRCA1 expression, *e.g.*, breast cancer.

In a preferred embodiment the iRNA agent silences mutations in the PTEN tumor suppressor gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted PTEN expression, *e.g.*, hamartomas, gliomas, and prostate and endometrial cancers.

In a preferred embodiment the iRNA agent silences MLL fusion genes, *e.g.*, MLL-AF9, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted MLL fusion gene expression, *e.g.*, acute leukemias.

In another preferred embodiment the iRNA agent silences the BCR/ABL fusion gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted BCR/ABL fusion gene expression, e.g., acute and chronic leukemias.

In another preferred embodiment the iRNA agent silences the TEL/AML1 fusion gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted TEL/AML1 fusion gene expression, e.g., childhood acute leukemia.

In another preferred embodiment the iRNA agent silences the EWS/FLI1 fusion gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted EWS/FLI1 fusion gene expression, e.g., Ewing Sarcoma.

In another preferred embodiment the iRNA agent silences the TLS/FUS1 fusion gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted TLS/FUS1 fusion gene expression, e.g., Myxoid liposarcoma.

In another preferred embodiment the iRNA agent silences the PAX3/FKHR fusion gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted PAX3/FKHR fusion gene expression, e.g., Myxoid liposarcoma.

In another preferred embodiment the iRNA agent silences the AML1/ETO fusion gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted AML1/ETO fusion gene expression, e.g., acute leukemia.

In another aspect, the invention features, a method of treating a subject, e.g., a human, at risk for or afflicted with a disease or disorder that may benefit by angiogenesis inhibition e.g., cancer. The method includes:

providing an iRNA agent, e.g., an iRNA agent having a structure described herein, which iRNA agent is homologous to and can silence, e.g., by cleavage, a gene which mediates angiogenesis;

administering the iRNA agent to a subject,

thereby treating the subject.

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In a preferred embodiment the iRNA agent silences the alpha v-integrin gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted alpha V integrin, e.g., brain tumors or tumors of epithelial origin.

In a preferred embodiment the iRNA agent silences the Flt-1 receptor gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted Flt-1 receptors, eg. Cancer and rheumatoid arthritis.

In a preferred embodiment the iRNA agent silences the tubulin gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted tubulin, eg. Cancer and retinal neovascularization.

In a preferred embodiment the iRNA agent silences the tubulin gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted tubulin, eg. Cancer and retinal neovascularization.

In another aspect, the invention features a method of treating a subject infected with a virus or at risk for or afflicted with a disorder or disease associated with a viral infection. The method includes:

providing an iRNA agent, e.g., and iRNA agent having a structure described herein, which iRNA agent is homologous to and can silence, e.g., by cleavage, a viral gene of a cellular gene which mediates viral function, e.g., entry or growth;

administering the iRNA agent to a subject, preferably a human subject,

thereby treating the subject.

Thus, the invention provides for a method of treating patients infected by the Human Papilloma Virus (HPV) or at risk for or afflicted with a disorder mediated by HPV, e.g, cervical cancer. HPV is linked to 95% of cervical carcinomas and thus an antiviral therapy is an attractive method to treat these cancers and other symptoms of viral infection.

In a preferred embodiment, the expression of a HPV gene is reduced. In another preferred embodiment, the HPV gene is one of the group of E2, E6, or E7.

In a preferred embodiment the expression of a human gene that is required for HPV replication is reduced.

The invention also includes a method of treating patients infected by the Human Immunodeficiency Virus (HIV) or at risk for or afflicted with a disorder mediated by HIV, *e.g.*, Acquired Immune Deficiency Syndrome (AIDS).

In a preferred embodiment, the expression of a HIV gene is reduced. In another preferred embodiment, the HIV gene is CCR5, Gag, or Rev.

In a preferred embodiment the expression of a human gene that is required for HIV replication is reduced. In another preferred embodiment, the gene is CD4 or Tsg101.

The invention also includes a method for treating patients infected by the Hepatitis B Virus (HBV) or at risk for or afflicted with a disorder mediated by HBV, *e.g.*, cirrhosis and heptocellular carcinoma.

In a preferred embodiment, the expression of a HBV gene is reduced. In another preferred embodiment, the targeted HBV gene encodes one of the group of the tail region of the HBV core protein, the pre-cregious (pre-c) region, or the cregious (c) region. In another preferred embodiment, a targeted HBV-RNA sequence is comprised of the poly(A) tail.

In preferred embodiment the expression of a human gene that is required for HBV replication is reduced.

The invention also provides for a method of treating patients infected by the Hepatitis A Virus (HAV), or at risk for or afflicted with a disorder mediated by HAV.

In a preferred embodiment the expression of a human gene that is required for HAV replication is reduced.

The present invention provides for a method of treating patients infected by the Hepatitis C Virus (HCV), or at risk for or afflicted with a disorder mediated by HCV, *e.g.*, cirrhosis

In a preferred embodiment, the expression of a HCV gene is reduced.

In another preferred embodiment the expression of a human gene that is required for HCV replication is reduced.

The present invention also provides for a method of treating patients infected by the any of the group of Hepatitis Viral strains comprising hepatitis D, E, F, G, or H, or patients at risk for or afflicted with a disorder mediated by any of these strains of hepatitis.

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In a preferred embodiment, the expression of a Hepatitis, D, E, F, G, or H gene is reduced.

In another preferred embodiment the expression of a human gene that is required for hepatitis D, E, F, G or H replication is reduced.

5   Methods of the invention also provide for treating patients infected by the Respiratory Syncytial Virus (RSV) or at risk for or afflicted with a disorder mediated by RSV, *e.g.,* lower respiratory tract infection in infants and childhood asthma, pneumonia and other complications, *e.g.,* in the elderly.

In a preferred embodiment, the expression of a RSV gene is reduced. In another
10   preferred embodiment, the targeted HBV gene encodes one of the group of genes N, L, or P.

In a preferred embodiment the expression of a human gene that is required for RSV replication is reduced.

Methods of the invention provide for treating patients infected by the Herpes Simplex Virus (HSV) or at risk for or afflicted with a disorder mediated by HSV, *e.g,* genital herpes and
15   cold sores as well as life-threatening or sight-impairing disease mainly in immunocompromised patients.

In a preferred embodiment, the expression of a HSV gene is reduced. In another preferred embodiment, the targeted HSV gene encodes DNA polymerase or the helicase-primase.

20   In a preferred embodiment the expression of a human gene that is required for HSV replication is reduced.

The invention also provides a method for treating patients infected by the herpes Cytomegalovirus (CMV) or at risk for or afflicted with a disorder mediated by CMV, *e.g.,* congenital virus infections and morbidity in immunocompromised patients.

5   In a preferred embodiment, the expression of a CMV gene is reduced.

In a preferred embodiment the expression of a human gene that is required for CMV replication is reduced.

Methods of the invention also provide for a method of treating patients infected by the herpes Epstein Barr Virus (EBV) or at risk for or afflicted with a disorder mediated by EBV, e.g., NK/T-cell lymphoma, non-Hodgkin lymphoma, and Hodgkin disease.

In a preferred embodiment, the expression of a EBV gene is reduced.

In a preferred embodiment the expression of a human gene that is required for EBV replication is reduced.

Methods of the invention also provide for treating patients infected by Kaposi's Sarcoma-associated Herpes Virus (KSHV), also called human herpesvirus 8, or patients at risk for or afflicted with a disorder mediated by KSHV, e.g., Kaposi's sarcoma, multicentric Castleman's disease and AIDS-associated primary effusion lymphoma.

In a preferred embodiment, the expression of a KSHV gene is reduced.

In a preferred embodiment the expression of a human gene that is required for KSHV replication is reduced.

The invention also includes a method for treating patients infected by the JC Virus (JCV) or a disease or disorder associated with this virus, e.g., progressive multifocal leukoencephalopathy (PML).

In a preferred embodiment, the expression of a JCV gene is reduced.

In preferred embodiment the expression of a human gene that is required for JCV replication is reduced.

Methods of the invention also provide for treating patients infected by the myxovirus or at risk for or afflicted with a disorder mediated by myxovirus, e.g., influenza.

In a preferred embodiment, the expression of a myxovirus gene is reduced.

In a preferred embodiment the expression of a human gene that is required for myxovirus replication is reduced.

Methods of the invention also provide for treating patients infected by the rhinovirus or at risk for of afflicted with a disorder mediated by rhinovirus, e.g., the common cold.

In a preferred embodiment, the expression of a rhinovirus gene is reduced.

In preferred embodiment the expression of a human gene that is required for rhinovirus replication is reduced.

Methods of the invention also provide for treating patients infected by the coronavirus or at risk for of afflicted with a disorder mediated by coronavirus, *e.g.*, the common cold.

5   In a preferred embodiment, the expression of a coronavirus gene is reduced.

In preferred embodiment the expression of a human gene that is required for coronavirus replication is reduced.

Methods of the invention also provide for treating patients infected by the flavivirus West Nile or at risk for or afflicted with a disorder mediated by West Nile Virus.

10   In a preferred embodiment, the expression of a West Nile Virus gene is reduced. In another preferred embodiment, the West Nile Virus gene is one of the group comprising E, NS3, or NS5.

In a preferred embodiment the expression of a human gene that is required for West Nile Virus replication is reduced.

15   Methods of the invention also provide for treating patients infected by the St. Louis Encephalitis flavivirus, or at risk for or afflicted with a disease or disorder associated with this virus, *e.g.*, viral haemorrhagic fever or neurological disease.

In a preferred embodiment, the expression of a St. Louis Encephalitis gene is reduced.

In a preferred embodiment the expression of a human gene that is required for St. Louis
20   Encephalitis virus replication is reduced.

Methods of the invention also provide for treating patients infected by the Tick-borne encephalitis flavivirus, or at risk for or afflicted with a disorder mediated by Tick-borne encephalitis virus, *e.g.*, viral haemorrhagic fever and neurological disease.

In a preferred embodiment, the expression of a Tick-borne encephalitis virus gene is
5   reduced.

In a preferred embodiment the expression of a human gene that is required for Tick-borne encephalitis virus replication is reduced.

Methods of the invention also provide for methods of treating patients infected by the Murray Valley encephalitis flavivirus, which commonly results in viral haemorrhagic fever and neurological disease.

In a preferred embodiment, the expression of a Murray Valley encephalitis virus gene is reduced.

In a preferred embodiment the expression of a human gene that is required for Murray Valley encephalitis virus replication is reduced.

The invention also includes methods for treating patients infected by the dengue flavivirus, or a disease or disorder associated with this virus, e.g., dengue haemorrhagic fever.

In a preferred embodiment, the expression of a dengue virus gene is reduced.

In a preferred embodiment the expression of a human gene that is required for dengue virus replication is reduced.

Methods of the invention also provide for treating patients infected by the Simian Virus 40 (SV40) or at risk for or afflicted with a disorder mediated by SV40, e.g., tumorigenesis.

In a preferred embodiment, the expression of a SV40 gene is reduced.

In a preferred embodiment the expression of a human gene that is required for SV40 replication is reduced.

The invention also includes methods for treating patients infected by the Human T Cell Lymphotropic Virus (HTLV), or a disease or disorder associated with this virus, e.g., leukemia and myelopathy.

In a preferred embodiment, the expression of a HTLV gene is reduced. In another preferred embodiment the HTLV1 gene is the Tax transcriptional activator.

In a preferred embodiment the expression of a human gene that is required for HTLV replication is reduced.

Methods of the invention also provide for treating patients infected by the Moloney-Murine Leukemia Virus (Mo-MuLV) or at risk for or afflicted with a disorder mediated by Mo-MuLV, e.g., T-cell leukemia.

In a preferred embodiment, the expression of a Mo-MuLV gene is reduced.

In a preferred embodiment the expression of a human gene that is required for Mo-MuLV replication is reduced.

Methods of the invention also provide for treating patients infected by the encephalomyocarditis virus (EMCV) or at risk for or afflicted with a disorder mediated by EMCV, *e.g.* myocarditis. EMCV leads to myocarditis in mice and pigs and is capable of infecting human myocardial cells. This virus is therefore a concern for patients undergoing xenotransplantation.

In a preferred embodiment, the expression of a EMCV gene is reduced.

In a preferred embodiment the expression of a human gene that is required for EMCV replication is reduced.

The invention also includes a method for treating patients infected by the measles virus (MV) or at risk for or afflicted with a disorder mediated by MV, *e.g.* measles.

In a preferred embodiment, the expression of a MV gene is reduced.

In a preferred embodiment the expression of a human gene that is required for MV replication is reduced.

The invention also includes a method for treating patients infected by the Vericella zoster virus (VZV) or at risk for or afflicted with a disorder mediated by VZV, *e.g.* chicken pox or shingles (also called zoster).

In a preferred embodiment, the expression of a VZV gene is reduced.

In a preferred embodiment the expression of a human gene that is required for VZV replication is reduced.

The invention also includes a method for treating patients infected by an adenovirus or at risk for or afflicted with a disorder mediated by an adenovirus, *e.g.* respiratory tract infection.

In a preferred embodiment, the expression of an adenovirus gene is reduced.

In a preferred embodiment the expression of a human gene that is required for adenovirus replication is reduced.

The invention includes a method for treating patients infected by a yellow fever virus (YFV) or at risk for or afflicted with a disorder mediated by a YFV, *e.g.* respiratory tract infection.

In a preferred embodiment, the expression of a YFV gene is reduced. In another preferred embodiment, the preferred gene is one of a group that includes the E, NS2A, or NS3 genes.

In a preferred embodiment the expression of a human gene that is required for YFV replication is reduced.

Methods of the invention also provide for treating patients infected by the poliovirus or at risk for or afflicted with a disorder mediated by poliovirus, *e.g.*, polio.

In a preferred embodiment, the expression of a poliovirus gene is reduced.

In a preferred embodiment the expression of a human gene that is required for poliovirus replication is reduced.

Methods of the invention also provide for treating patients infected by a poxvirus or at risk for or afflicted with a disorder mediated by a poxvirus, *e.g.*, smallpox

In a preferred embodiment, the expression of a poxvirus gene is reduced.

In a preferred embodiment the expression of a human gene that is required for poxvirus replication is reduced.

In another, aspect the invention features methods of treating a subject infected with a pathogen, *e.g.*, a bacterial, amoebic, parasitic, or fungal pathogen. The method includes:

providing a iRNA agent, *e.g.*, a siRNA having a structure described herein, where siRNA is homologous to and can silence, *e.g.*, by cleavage of a pathogen gene;

administering the iRNA agent to a subject, prefereably a human subject,

thereby treating the subject.

The target gene can be one involved in growth, cell wall synthesis, protein synthesis, transcription, energy metabolism, *e.g.*, the Krebs cycle, or toxin production.

Thus, the present invention provides for a method of treating patients infected by a plasmodium that causes malaria.

In a preferred embodiment, the expression of a plasmodium gene is reduced. In another preferred embodiment, the gene is apical membrane antigen 1 (AMA1).

In a preferred embodiment the expression of a human gene that is required for plasmodium replication is reduced.

The invention also includes methods for treating patients infected by the Mycobacterium ulcerans, or a disease or disorder associated with this pathogen, *e.g.* Buruli ulcers.

In a preferred embodiment, the expression of a Mycobacterium ulcerans gene is reduced.

In a preferred embodiment the expression of a human gene that is required for Mycobacterium ulcerans replication is reduced.

The invention also includes methods for treating patients infected by the Mycobacterium tuberculosis, or a disease or disorder associated with this pathogen, *e.g.* tuberculosis.

In a preferred embodiment, the expression of a Mycobacterium tuberculosis gene is reduced.

In a preferred embodiment the expression of a human gene that is required for Mycobacterium tuberculosis replication is reduced.

The invention also includes methods for treating patients infected by the Mycobacterium leprae, or a disease or disorder associated with this pathogen, *e.g.* leprosy.

In a preferred embodiment, the expression of a Mycobacterium leprae gene is reduced.

In a preferred embodiment the expression of a human gene that is required for Mycobacterium leprae replication is reduced.

The invention also includes methods for treating patients infected by the bacteria Staphylococcus aureus, or a disease or disorder associated with this pathogen, *e.g.* infections of the skin and muscous membranes.

In a preferred embodiment, the expression of a Staphylococcus aureus gene is reduced.

In a preferred embodiment the expression of a human gene that is required for Staphylococcus aureus replication is reduced.

The invention also includes methods for treating patients infected by the bacteria Streptococcus pneumoniae, or a disease or disorder associated with this pathogen, *e.g.* pneumonia or childhood lower respiratory tract infection.

In a preferred embodiment, the expression of a Streptococcus pneumoniae gene is reduced.

In a preferred embodiment the expression of a human gene that is required for Streptococcus pneumoniae replication is reduced.

The invention also includes methods for treating patients infected by the bacteria Streptococcus pyogenes, or a disease or disorder associated with this pathogen, *e.g.* Strep throat or Scarlet fever.

In a preferred embodiment, the expression of a Streptococcus pyogenes gene is reduced.

In a preferred embodiment the expression of a human gene that is required for Streptococcus pyogenes replication is reduced.

The invention also includes methods for treating patients infected by the bacteria Chlamydia pneumoniae, or a disease or disorder associated with this pathogen, *e.g.* pneumonia or childhood lower respiratory tract infection

In a preferred embodiment, the expression of a Chlamydia pneumoniae gene is reduced.

In a preferred embodiment the expression of a human gene that is required for Chlamydia pneumoniae replication is reduced.

The invention also includes methods for treating patients infected by the bacteria Mycoplasma pneumoniae, or a disease or disorder associated with this pathogen, *e.g.* pneumonia or childhood lower respiratory tract infection

In a preferred embodiment, the expression of a Mycoplasma pneumoniae gene is reduced.

In a preferred embodiment the expression of a human gene that is required for Mycoplasma pneumoniae replication is reduced.

In one aspect, the invention features, a method of treating a subject, *e.g.*, a human, at risk for or afflicted with a disease or disorder characterized by an unwanted immune response, *e.g.*, an inflammatory disease or disorder, or an autoimmune disease or disorder.   The method includes:

5   providing an iRNA agent, *e.g.*, an iRNA agent having a structure described herein, which iRNA agent is homologous to and can silence, *e.g.*, by cleavage, a gene which mediates an unwanted immune response;

administering the iRNA agent to a subject,

thereby treating the subject.

10   In a preferred embodiment the disease or disorder is an ischemia or reperfusion injury, *e.g.*, ischemia or reperfusion injury associated with acute myocardial infarction, unstable angina, cardiopulmonary bypass, surgical intervention *e.g.*, angioplasty, *e.g.*, percutaneous transluminal coronary angioplasty, the response to a transplantated organ or tissue, *e.g.*, transplanted cardiac or vascular tissue; or thrombolysis.

15   In a preferred embodiment the disease or disorder is restenosis, *e.g.*, restenosis associated with surgical intervention *e.g.*, angioplasty, *e.g.*, percutaneous transluminal coronary angioplasty.

In a preferred embodiment the disease or disorder is Inflammatory Bowel Disease, *e.g.*, Crohn Disease or Ulcerative Colitis.

In a preferred embodiment the disease or disorder is inflammation associated with an
20 infection or injury.

In a preferred embodiment the disease or disorder is asthma, lupus, multiple sclerosis, diabetes, *e.g.*, type II diabetes, arthritis, *e.g.*, rheumatoid or psoriatic.

In particularly preferred embodiments the iRNA agent silences an integrin or co-ligand thereof, *e.g.*, VLA4, VCAM, ICAM.

25   In particularly preferred embodiments the iRNA agent silences a selectin or co-ligand thereof, *e.g.*, P-selectin, E-selectin (ELAM), I-selectin, P-selectin glycoprotein-1 (PSGL-1).

In particularly preferred embodiments the iRNA agent silences a component of the complement system, *e.g.*, C3, C5, C3aR, C5aR, C3 convertase, C5 convertase.

In particularly preferred embodiments the iRNA agent silences a chemokine or receptor thereof, e.g., TNFI, TNFJ, IL-1I, IL-1J, IL-2, IL-2R, IL-4, IL-4R, IL-5, IL-6, IL-8, TNFRI, TNFRII, IgE, SCYA11, CCR3.

In other embodiments the iRNA agent silences GCSF, Gro1, Gro2, Gro3, PF4, MIG, Pro-Platelet Basic Protein (PPBP), MIP-1I, MIP-1J, RANTES, MCP-1, MCP-2, MCP-3, CMBKR1, CMBKR2, CMBKR3, CMBKR5, AIF-1, I-309.

In one aspect, the invention features, a method of treating a subject, e.g., a human, at risk for or afflicted with acute pain or chronic pain. The method includes:

providing an iRNA agent, which iRNA is homologous to and can silence, e.g., by cleavage, a gene which mediates the processing of pain;

administering the iRNA to a subject,

thereby treating the subject.

In particularly preferred embodiments the iRNA agent silences a component of an ion channel.

In particularly preferred embodiments the iRNA agent silences a neurotransmitter receptor or ligand.

In one aspect, the invention features, a method of treating a subject, e.g., a human, at risk for or afflicted with a neurological disease or disorder. The method includes:

providing an iRNA agent which iRNA is homologous to and can silence, e.g., by cleavage, a gene which mediates a neurological disease or disorder;

administering the iRNA agent to a subject,

thereby treating the subject.

In a preferred embodiment the disease or disorder is Alzheimer's Disease or Parkinson Disease.

In particularly preferred embodiments the iRNA agent silences an amyloid-family gene, e.g., APP; a presenilin gene, e.g., PSEN1 and PSEN2, or I-synuclein.

In a preferred embodiment the disease or disorder is a neurodegenerative trinucleotide repeat disorder, e.g., Huntington disease, dentatorubral pallidoluysian atrophy or a spinocerebellar ataxia, e.g., SCA1, SCA2, SCA3 (Machado-Joseph disease), SCA7 or SCA8.

In particularly preferred embodiments the iRNA agent silences HD, DRPLA, SCA1, SCA2, MJD1, CACNL1A4, SCA7, SCA8.

The loss of heterozygosity (LOH) can result in hemizygosity for sequence, e.g., genes, in the area of LOH. This can result in a significant genetic difference between normal and disease-state cells, e.g., cancer cells, and provides a useful difference between normal and disease-state cells, e.g., cancer cells. This difference can arise because a gene or other sequence is heterozygous in euploid cells but is hemizygous in cells having LOH. The regions of LOH will often include a gene, the loss of which promotes unwanted proliferation, e.g., a tumor suppressor gene, and other sequences including, e.g., other genes, in some cases a gene which is essential for normal function, e.g., growth. Methods of the invention rely, in part, on the specific cleavage or silencing of one allele of an essential gene with an iRNA agent of the invention. The iRNA agent is selected such that it targets the single allele of the essential gene found in the cells having LOH but does not silence the other allele, which is present in cells which do not show LOH. In essence, it discriminates between the two alleles, preferentially silencing the selected allele. In essence polymorphisms, e.g., SNPs of essential genes that are affected by LOH, are used as a target for a disorder characterized by cells having LOH, e.g., cancer cells having LOH.

E.g., one of ordinary skill in the art can identify essential genes which are in proximity to tumor suppressor genes, and which are within a LOH region which includes the tumor suppressor gene. The gene encoding the large subunit of human RNA polymerase II, POLR2A, a gene located in close proximity to the tumor suppressor gene p53, is such a gene. It frequently occurs within a region of LOH in cancer cells. Other genes that occur within LOH regions and are lost in many cancer cell types include the group comprising replication protein A 70-kDa subunit, replication protein A 32-kD, ribonucleotide reductase, thymidilate synthase, TATA associated factor 2H, ribosomal protein S14, eukaryotic initiation factor 5A, alanyl tRNA synthetase, cysteinyl tRNA synthetase, NaK ATPase, alpha-1 subunit, and transferrin receptor.

Accordingly, the invention features, a method of treating a disorder characterized by LOH, e.g., cancer. The method includes:

optionally, determining the genotype of the allele of a gene in the region of LOH and preferably determining the genotype of both alleles of the gene in a normal cell;

providing an iRNA agent which preferentially cleaves or silences the allele found in the LOH cells;

administering the iRNA to the subject,

thereby treating the disorder.

The invention also includes a iRNA agent disclosed herein, *e.g*, an iRNA agent which can preferentially silence, *e.g.*, cleave, one allele of a polymorphic gene

In another aspect, the invention provides a method of cleaving or silencing more than one gene with an iRNA agent. In these embodiments the iRNA agent is selected so that it has sufficient homology to a sequence found in more than one gene. For example, the sequence AAGCTGGCCCTGGACATGGAGAT (SEQ ID NO:55) is conserved between mouse lamin B1, lamin B2, keratin complex 2-gene 1 and lamin A/C. Thus an iRNA agent targeted to this sequence would effectively silence the entire collection of genes.

The invention also includes an iRNA agent disclosed herein, which can silence more than one gene.

ROUTE OF DELIVERY

For ease of exposition the formulations, compositions and methods in this section are discussed largely with regard to unmodified iRNA agents. It should be understood, however, that these formulations, compositions and methods can be practiced with other iRNA agents, *e.g.*, modified iRNA agents, and such practice is within the invention. A composition that includes a iRNA can be delivered to a subject by a variety of routes. Exemplary routes include: intravenous, topical, rectal, anal, vaginal, nasal, pulmonary, ocular.

The iRNA molecules of the invention can be incorporated into pharmaceutical compositions suitable for administration. Such compositions typically include one or more species of iRNA and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for

pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic, vaginal, rectal, intranasal, transdermal), oral or parenteral. Parenteral administration includes intravenous drip, subcutaneous, intraperitoneal or intramuscular injection, or intrathecal or intraventricular administration.

The route and site of administration may be chosen to enhance targeting. For example, to target muscle cells, intramuscular injection into the muscles of interest would be a logical choice. Lung cells might be targeted by administering the iRNA in aerosol form. The vascular endothelial cells could be targeted by coating a balloon catheter with the iRNA and mechanically introducing the DNA.

Formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful.

Compositions for oral administration include powders or granules, suspensions or solutions in water, syrups, elixirs or non-aqueous media, tablets, capsules, lozenges, or troches. In the case of tablets, carriers that can be used include lactose, sodium citrate and salts of phosphoric acid. Various disintegrants such as starch, and lubricating agents such as magnesium stearate, sodium lauryl sulfate and talc, are commonly used in tablets. For oral administration in capsule form, useful diluents are lactose and high molecular weight polyethylene glycols. When aqueous suspensions are required for oral use, the nucleic acid compositions can be combined with emulsifying and suspending agents. If desired, certain sweetening and/or flavoring agents can be added.

Compositions for intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives.

Formulations for parenteral administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives. Intraventricular injection may be

facilitated by an intraventricular catheter, for example, attached to a reservoir. For intravenous use, the total concentration of solutes should be controlled to render the preparation isotonic.

For ocular administration, ointments or droppable liquids may be delivered by ocular delivery systems known to the art such as applicators or eye droppers. Such compositions can include mucomimetics such as hyaluronic acid, chondroitin sulfate, hydroxypropyl methylcellulose or poly(vinyl alcohol), preservatives such as sorbic acid, EDTA or benzylchronium chloride, and the usual quantities of diluents and/or carriers.

Topical Delivery

For ease of exposition the formulations, compositions and methods in this section are discussed largely with regard to unmodified iRNA agents. It should be understood, however, that these formulations, compositions and methods can be practiced with other iRNA agents, e.g., modified iRNA agents, and such practice is within the invention. In a preferred embodiment, an iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, (e.g., a precursor, e.g., a larger iRNA agent which can be processed into a sRNA agent, or a DNA which encodes an iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, or precursor thereof) is delivered to a subject via topical administration. "Topical administration" refers to the delivery to a subject by contacting the formulation directly to a surface of the subject. The most common form of topical delivery is to the skin, but a composition disclosed herein can also be directly applied to other surfaces of the body, e.g., to the eye, a mucous membrane, to surfaces of a body cavity or to an internal surface. As mentioned above, the most common topical delivery is to the skin. The term encompasses several routes of administration including, but not limited to, topical and transdermal. These modes of administration typically include penetration of the skin's permeability barrier and efficient delivery to the target tissue or stratum. Topical administration can be used as a means to penetrate the epidermis and dermis and ultimately achieve systemic delivery of the composition. Topical administration can also be used as a means to selectively deliver oligonucleotides to the epidermis or dermis of a subject, or to specific strata thereof, or to an underlying tissue.

The term "skin," as used herein, refers to the epidermis and/or dermis of an animal. Mammalian skin consists of two major, distinct layers. The outer layer of the skin is called the epidermis. The epidermis is comprised of the stratum corneum, the stratum granulosum, the stratum spinosum, and the stratum basale, with the stratum corneum being at the surface of the

skin and the stratum basale being the deepest portion of the epidermis. The epidermis is between 50 μm and 0.2 mm thick, depending on its location on the body.

Beneath the epidermis is the dermis, which is significantly thicker than the epidermis. The dermis is primarily composed of collagen in the form of fibrous bundles. The collagenous bundles provide support for, inter alia, blood vessels, lymph capillaries, glands, nerve endings and immunologically active cells.

One of the major functions of the skin as an organ is to regulate the entry of substances into the body. The principal permeability barrier of the skin is provided by the stratum corneum, which is formed from many layers of cells in various states of differentiation. The spaces between cells in the stratum corneum is filled with different lipids arranged in lattice-like formations that provide seals to further enhance the skins permeability barrier.

The permeability barrier provided by the skin is such that it is largely impermeable to molecules having molecular weight greater than about 750 Da. For larger molecules to cross the skin's permeability barrier, mechanisms other than normal osmosis must be used.

Several factors determine the permeability of the skin to administered agents. These factors include the characteristics of the treated skin, the characteristics of the delivery agent, interactions between both the drug and delivery agent and the drug and skin, the dosage of the drug applied, the form of treatment, and the post treatment regimen. To selectively target the epidermis and dermis, it is sometimes possible to formulate a composition that comprises one or more penetration enhancers that will enable penetration of the drug to a preselected stratum.

Transdermal delivery is a valuable route for the administration of lipid soluble therapeutics. The dermis is more permeable than the epidermis and therefore absorption is much more rapid through abraded, burned or denuded skin. Inflammation and other physiologic conditions that increase blood flow to the skin also enhance transdermal adsorption. Absorption via this route may be enhanced by the use of an oily vehicle (inunction) or through the use of one or more penetration enhancers. Other effective ways to deliver a composition disclosed herein via the transdermal route include hydration of the skin and the use of controlled release topical patches. The transdermal route provides a potentially effective means to deliver a composition disclosed herein for systemic and/or local therapy.

In addition, iontophoresis (transfer of ionic solutes through biological membranes under the influence of an electric field) (Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier

Systems, 1991, p. 163), phonophoresis or sonophoresis (use of ultrasound to enhance the absorption of various therapeutic agents across biological membranes, notably the skin and the cornea) (Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p. 166), and optimization of vehicle characteristics relative to dose position and retention at the site of administration (Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p. 168) may be useful methods for enhancing the transport of topically applied compositions across skin and mucosal sites.

The compositions and methods provided may also be used to examine the function of various proteins and genes *in vitro* in cultured or preserved dermal tissues and in animals. The invention can be thus applied to examine the function of any gene. The methods of the invention can also be used therapeutically or prophylactically. For example, for the treatment of animals that are known or suspected to suffer from diseases such as psoriasis, lichen planus, toxic epidermal necrolysis, ertythema multiforme, basal cell carcinoma, squamous cell carcinoma, malignant melanoma, Paget's disease, Kaposi's sarcoma, pulmonary fibrosis, Lyme disease and viral, fungal and bacterial infections of the skin.

Pulmonary Delivery

For ease of exposition the formulations, compositions and methods in this section are discussed largely with regard to unmodified iRNA agents. It should be understood, however, that these formulations, compositions and methods can be practiced with other iRNA agents, *e.g.*, modified iRNA agents, and such practice is within the invention. A composition that includes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or sRNA agent, (*e.g.*, a precursor, *e.g.*, a larger iRNA agent which can be processed into a sRNA agent, or a DNA which encodes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or sRNA agent, or precursor thereof) can be administered to a subject by pulmonary delivery. Pulmonary delivery compositions can be delivered by inhalation by the patient of a dispersion so that the composition, preferably iRNA, within the dispersion can reach the lung where it can be readily absorbed through the alveolar region directly into blood circulation. Pulmonary delivery can be effective both for systemic delivery and for localized delivery to treat diseases of the lungs.

Pulmonary delivery can be achieved by different approaches, including the use of nebulized, aerosolized, micellular and dry powder-based formulations. Delivery can be achieved with liquid nebulizers, aerosol-based inhalers, and dry powder dispersion devices. Metered-dose devices are preferred. One of the benefits of using an atomizer or inhaler is that the potential for

contamination is minimized because the devices are self contained. Dry powder dispersion devices, for example, deliver drugs that may be readily formulated as dry powders. A iRNA composition may be stably stored as lyophilized or spray-dried powders by itself or in combination with suitable powder carriers. The delivery of a composition for inhalation can be mediated by a dosing timing element which can include a timer, a dose counter, time measuring device, or a time indicator which when incorporated into the device enables dose tracking, compliance monitoring, and/or dose triggering to a patient during administration of the aerosol medicament.

The term "powder" means a composition that consists of finely dispersed solid particles that are free flowing and capable of being readily dispersed in an inhalation device and subsequently inhaled by a subject so that the particles reach the lungs to permit penetration into the alveoli. Thus, the powder is said to be "respirable." Preferably the average particle size is less than about 10 μm in diameter preferably with a relatively uniform spheroidal shape distribution. More preferably the diameter is less than about 7.5 μm and most preferably less than about 5.0 μm. Usually the particle size distribution is between about 0.1 μm and about 5 μm in diameter, particularly about 0.3 μm to about 5 μm.

The term "dry" means that the composition has a moisture content below about 10% by weight (% w) water, usually below about 5% w and preferably less it than about 3% w. A dry composition can be such that the particles are readily dispersible in an inhalation device to form an aerosol.

The term "therapeutically effective amount" is the amount present in the composition that is needed to provide the desired level of drug in the subject to be treated to give the anticipated physiological response.

The term "physiologically effective amount" is that amount delivered to a subject to give the desired palliative or curative effect.

The term "pharmaceutically acceptable carrier" means that the carrier can be taken into the lungs with no significant adverse toxicological effects on the lungs.

The types of pharmaceutical excipients that are useful as carrier include stabilizers such as human serum albumin (HSA), bulking agents such as carbohydrates, amino acids and polypeptides; pH adjusters or buffers; salts such as sodium chloride; and the like. These carriers may be in a crystalline or amorphous form or may be a mixture of the two.

Bulking agents that are particularly valuable include compatible carbohydrates, polypeptides, amino acids or combinations thereof. Suitable carbohydrates include monosaccharides such as galactose, D-mannose, sorbose, and the like; disaccharides, such as lactose, trehalose, and the like; cyclodextrins, such as 2-hydroxypropyl-.beta.-cyclodextrin; and polysaccharides, such as raffinose, maltodextrins, dextrans, and the like; alditols, such as mannitol, xylitol, and the like. A preferred group of carbohydrates includes lactose, trehalose, raffinose maltodextrins, and mannitol. Suitable polypeptides include aspartame. Amino acids include alanine and glycine, with glycine being preferred.

Additives, which are minor components of the composition of this invention, may be included for conformational stability during spray drying and for improving dispersibility of the powder. These additives include hydrophobic amino acids such as tryptophan, tyrosine, leucine, phenylalanine, and the like.

Suitable pH adjusters or buffers include organic salts prepared from organic acids and bases, such as sodium citrate, sodium ascorbate, and the like; sodium citrate is preferred.

Pulmonary administration of a micellar iRNA formulation may be achieved through metered dose spray devices with propellants such as tetrafluoroethane, heptafluoroethane, dimethylfluoropropane, tetrafluoropropane, butane, isobutane, dimethyl ether and other non-CFC and CFC propellants.

Oral or Nasal Delivery

For ease of exposition the formulations, compositions and methods in this section are discussed largely with regard to unmodified iRNA agents. It should be understood, however, that these formulations, compositions and methods can be practiced with other iRNA agents, e.g., modified iRNA agents, and such practice is within the invention. Both the oral and nasal membranes offer advantages over other routes of administration. For example, drugs administered through these membranes have a rapid onset of action, provide therapeutic plasma levels, avoid first pass effect of hepatic metabolism, and avoid exposure of the drug to the hostile gastrointestinal (GI) environment. Additional advantages include easy access to the membrane sites so that the drug can be applied, localized and removed easily.

In oral delivery, compositions can be targeted to a surface of the oral cavity, e.g., to sublingual mucosa which includes the membrane of ventral surface of the tongue and the floor of the mouth or the buccal mucosa which constitutes the lining of the cheek. The sublingual mucosa

is relatively permeable thus giving rapid absorption and acceptable bioavailability of many drugs. Further, the sublingual mucosa is convenient, acceptable and easily accessible.

The ability of molecules to permeate through the oral mucosa appears to be related to molecular size, lipid solubility and peptide protein ionization. Small molecules, less than 1000 daltons appear to cross mucosa rapidly. As molecular size increases, the permeability decreases rapidly. Lipid soluble compounds are more permeable than non-lipid soluble molecules. Maximum absorption occurs when molecules are un-ionized or neutral in electrical charges. Therefore charged molecules present the biggest challenges to absorption through the oral mucosae.

A pharmaceutical composition of iRNA may also be administered to the buccal cavity of a human being by spraying into the cavity, without inhalation, from a metered dose spray dispenser, a mixed micellar pharmaceutical formulation as described above and a propellant. In one embodiment, the dispenser is first shaken prior to spraying the pharmaceutical formulation and propellant into the buccal cavity.

Devices

For ease of exposition the devices, formulations, compositions and methods in this section are discussed largely with regard to unmodified iRNA agents. It should be understood, however, that these devices, formulations, compositions and methods can be practiced with other iRNA agents, e.g., modified iRNA agents, and such practice is within the invention. An iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, (e.g., a precursor, e.g., a larger iRNA agent which can be processed into a sRNA agent, or a DNA which encodes an iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, or precursor thereof) can be disposed on or in a device, e.g., a device which implanted or otherwise placed in a subject. Exemplary devices include devices which are introduced into the vasculature, e.g., devices inserted into the lumen of a vascular tissue, or which devices themselves form a part of the vasculature, including stents, catheters, heart valves, and other vascular devices. These devices, e.g., catheters or stents, can be placed in the vasculature of the lung, heart, or leg.

Other devices include non-vascular devices, e.g., devices implanted in the peritoneum, or in organ or glandular tissue, e.g., artificial organs. The device can release a therapeutic substance in addition to a iRNA, e.g., a device can release insulin.

Other devices include artificial joints, e.g., hip joints, and other orthopedic implants.

In one embodiment, unit doses or measured doses of a composition that includes iRNA are dispensed by an implanted device. The device can include a sensor that monitors a parameter within a subject. For example, the device can include pump, *e.g.*, and, optionally, associated electronics.

5   Tissue, *e.g.*, cells or organs, such as the kidney, can be treated with An iRNA agent, *e.g.*, a double-stranded iRNA agent, or sRNA agent, (*e.g.*, a precursor, *e.g.*, a larger iRNA agent which can be processed into a sRNA agent, or a DNA which encodes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or sRNA agent, or precursor thereof) ex vivo and then administered or implanted in a subject.

10   The tissue can be autologous, allogeneic, or xenogeneic tissue. For example, tissue (e.g., kidney) can be treated to reduce graft v. host disease. In other embodiments, the tissue is allogeneic and the tissue is treated to treat a disorder characterized by unwanted gene expression in that tissue, such as in the kidney. In another example, tissue containing hematopoietic cells, *e.g.*, bone marrow hematopoietic cells, can be treated to inhibit unwanted cell proliferation.

15   Introduction of treated tissue, whether autologous or transplant, can be combined with other therapies.

       In some implementations, the iRNA treated cells are insulated from other cells, *e.g.*, by a semi-permeable porous barrier that prevents the cells from leaving the implant, but enables molecules from the body to reach the cells and molecules produced by the cells to enter the body.
20   In one embodiment, the porous barrier is formed from alginate.

       In one embodiment, a contraceptive device is coated with or contains an iRNA agent, *e.g.*, a double-stranded iRNA agent, or sRNA agent, (*e.g.*, a precursor, *e.g.*, a larger iRNA agent which can be processed into a sRNA agent, or a DNA which encodes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or sRNA agent, or precursor thereof). Exemplary devices include
25   condoms, diaphragms, IUD (implantable uterine devices, sponges, vaginal sheaths, and birth control devices. In one embodiment, the iRNA is chosen to inactive sperm or egg. In another embodiment, the iRNA is chosen to be complementary to a viral or pathogen RNA, *e.g.*, an RNA of an STD. In some instances, the iRNA composition can include a spermicide.

DOSAGE

In one aspect, the invention features a method of administering an iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, to a subject (e.g., a human subject). The method includes administering a unit dose of the iRNA agent, e.g., a sRNA agent, e.g., double stranded

5   sRNA agent that (a) the double-stranded part is 19-25 nucleotides (nt) long, preferably 21-23 nt, (b) is complementary to a target RNA (e.g., an endogenous or pathogen target RNA), and, optionally, (c) includes at least one 3' overhang 1-5 nucleotide long. In one embodiment, the unit dose is less than 1.4 mg per kg of bodyweight, or less than 10, 5, 2, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00005 or 0.00001 mg per kg of bodyweight, and less than 200 nmole of

10   RNA agent (e.g. about 4.4×10^{16} copies) per kg of bodyweight, or less than 1500, 750, 300, 150, 75, 15, 7.5, 1.5, 0.75, 0.15, 0.075, 0.015, 0.0075, 0.0015, 0.00075, 0.00015 nmole of RNA agent per kg of bodyweight.

The defined amount can be an amount effective to treat or prevent a disease or disorder, e.g., a disease or disorder associated with the target RNA, such as an RNA present in the kidney.

15   The unit dose, for example, can be administered by injection (e.g., intravenous or intramuscular), an inhaled dose, or a topical application. Particularly preferred dosages are less than 2, 1, or 0.1 mg/kg of body weight.

In a preferred embodiment, the unit dose is administered less frequently than once a day, e.g., less than every 2, 4, 8 or 30 days. In another embodiment, the unit dose is not administered

20   with a frequency (e.g., not a regular frequency). For example, the unit dose may be administered a single time.

In one embodiment, the effective dose is administered with other traditional therapeutic modalities. In one embodiment, the subject has a viral infection and the modality is an antiviral agent other than an iRNA agent, e.g., other than a double-stranded iRNA agent, or sRNA agent,.

5   In another embodiment, the subject has atherosclerosis and the effective dose of an iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, is administered in combination with, e.g., after surgical intervention, e.g., angioplasty.

In one embodiment, a subject is administered an initial dose and one or more maintenance doses of an iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, (e.g.,

）   a precursor, e.g., a larger iRNA agent which can be processed into a sRNA agent, or a DNA which encodes an iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, or precursor

thereof). The maintenance dose or doses are generally lower than the initial dose, e.g., one-half less of the initial dose. A maintenance regimen can include treating the subject with a dose or doses ranging from 0.01 μg to 1.4 mg/kg of body weight per day, e.g., 10, 1, 0.1, 0.01, 0.001, or 0.00001 mg per kg of bodyweight per day. The maintenance doses are preferably administered no more than once every 5, 10, or 30 days. Further, the treatment regimen may last for a period of time which will vary depending upon the nature of the particular disease, its severity and the overall condition of the patient. In preferred embodiments the dosage may be delivered no more than once per day, e.g., no more than once per 24, 36, 48, or more hours, e.g., no more than once for every 5 or 8 days. Following treatment, the patient can be monitored for changes in his condition and for alleviation of the symptoms of the disease state. The dosage of the compound may either be increased in the event the patient does not respond significantly to current dosage levels, or the dose may be decreased if an alleviation of the symptoms of the disease state is observed, if the disease state has been ablated, or if undesired side-effects are observed.

The effective dose can be administered in a single dose or in two or more doses, as desired or considered appropriate under the specific circumstances. If desired to facilitate repeated or frequent infusions, implantation of a delivery device, e.g., a pump, semi-permanent stent (e.g., intravenous, intraperitoneal, intracisternal or intracapsular), or reservoir may be advisable.

In one embodiment, the iRNA agent pharmaceutical composition includes a plurality of iRNA agent species. In another embodiment, the iRNA agent species has sequences that are non-overlapping and non-adjacent to another species with respect to a naturally occurring target sequence. In another embodiment, the plurality of iRNA agent species is specific for different naturally occurring target genes. In another embodiment, the iRNA agent is allele specific.

In some cases, a patient is treated with a iRNA agent in conjunction with other therapeutic modalities. For example, a patient being treated for a kidney disease, e.g., early stage renal disease, can be administered an iRNA agent specific for a target gene known to enhance the progression of the disease in conjunction with a drug known to inhibit activity of the target gene product. For example, a patient who has early stage renal disease can be treated with an iRNA agent that targets an SGLT2 RNA, in conjunction with the small molecule phlorizin, which is known to block sodium-glucose cotransport and to subsequently reduce single nephron glomerular filtration rate. In another example, a patient being treated for a cancer of the kidney

can be administered an iRNA agent specific for a target essential for tumor cell proliferation in conjunction with a chemotherapy.

Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the compound of the invention is administered in maintenance doses, ranging from 0.01 μg to 100 g per kg of body weight (see US 6,107,094).

The concentration of the iRNA agent composition is an amount sufficient to be effective in treating or preventing a disorder or to regulate a physiological condition in humans. The concentration or amount of iRNA agent administered will depend on the parameters determined for the agent and the method of administration, e.g. nasal, buccal, pulmonary. For example, nasal formulations tend to require much lower concentrations of some ingredients in order to avoid irritation or burning of the nasal passages. It is sometimes desirable to dilute an oral formulation up to 10-100 times in order to provide a suitable nasal formulation.

Certain factors may influence the dosage required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of an iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, (e.g., a precursor, e.g., a larger iRNA agent which can be processed into a sRNA agent, or a DNA which encodes an iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, or precursor thereof) can include a single treatment or, preferably, can include a series of treatments. It will also be appreciated that the effective dosage of a iRNA agent such as a sRNA agent used for treatment may increase or decrease over the course of a particular treatment. Changes in dosage may result and become apparent from the results of diagnostic assays as described herein. For example, the subject can be monitored after administering a iRNA agent composition. Based on information from the monitoring, an additional amount of the iRNA agent composition can be administered.

Dosing is dependent on severity and responsiveness of the disease condition to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual compounds, and can generally be

estimated based on EC50s found to be effective in *in vitro* and *in vivo* animal models. In some embodiments, the animal models include transgenic animals that express a human gene, *e.g.* a gene that produces a target RNA. The transgenic animal can be deficient for the corresponding endogenous RNA. In another embodiment, the composition for testing includes a iRNA agent that is complementary, at least in an internal region, to a sequence that is conserved between the target RNA in the animal model and the target RNA in a human.

The inventors have discovered that iRNA agents described herein can be administered to mammals, particularly large mammals such as nonhuman primates or humans in a number of ways.

In one embodiment, the administration of the iRNA agent, *e.g.*, a double-stranded iRNA agent, or sRNA agent, composition is parenteral, *e.g.* intravenous (*e.g.*, as a bolus or as a diffusible infusion), intradermal, intraperitoneal, intramuscular, intrathecal, intraventricular, intracranial, subcutaneous, transmucosal, buccal, sublingual, endoscopic, rectal, oral, vaginal, topical, pulmonary, intranasal, urethral or ocular. Administration can be provided by the subject or by another person, *e.g.*, a health care provider. The medication can be provided in measured doses or in a dispenser which delivers a metered dose. Selected modes of delivery are discussed in more detail below.

The invention provides methods, compositions, and kits, for rectal administration or delivery of iRNA agents described herein.

Accordingly, an iRNA agent, *e.g.*, a double-stranded iRNA agent, or sRNA agent, (*e.g.*, a precursor, *e.g.*, a larger iRNA agent which can be processed into a sRNA agent , or a DNA which encodes a an iRNA agent, *e.g.*, a double-stranded iRNA agent, or sRNA agent, or precursor thereof) described herein, *e.g.*, a therapeutically effective amount of a iRNA agent described herein, *e.g.*, a iRNA agent having a double stranded region of less than 40, and preferably less than 30 nucleotides and having one or two 1-3 nucleotide single strand 3' overhangs can be administered rectally, *e.g.*, introduced through the rectum into the lower or upper colon. This approach is particularly useful in the treatment of, inflammatory disorders, disorders characterized by unwanted cell proliferation, *e.g.*, polyps, or colon cancer.

The medication can be delivered to a site in the colon by introducing a dispensing device, *e.g.*, a flexible, camera-guided device similar to that used for inspection of the colon or removal of polyps, which includes means for delivery of the medication.

The rectal administration of the iRNA agent is by means of an enema. The iRNA agent of the enema can be dissolved in a saline or buffered solution. The rectal administration can also by means of a suppository, which can include other ingredients, e.g., an excipient, e.g., cocoa butter or hydropropylmethylcellulose.

Any of the iRNA agents described herein can be administered orally, e.g., in the form of tablets, capsules, gel capsules, lozenges, troches or liquid syrups. Further, the composition can be applied topically to a surface of the oral cavity.

Any of the iRNA agents described herein can be administered buccally. For example, the medication can be sprayed into the buccal cavity or applied directly, e.g., in a liquid, solid, or gel form to a surface in the buccal cavity. This administration is particularly desirable for the treatment of inflammations of the buccal cavity, e.g., the gums or tongue, e.g., in one embodiment, the buccal administration is by spraying into the cavity, e.g., without inhalation, from a dispenser, e.g., a metered dose spray dispenser that dispenses the pharmaceutical composition and a propellant.

Any of the iRNA agents described herein can be administered to ocular tissue. For example, the medications can be applied to the surface of the eye or nearby tissue, e.g., the inside of the eyelid. They can be applied topically, e.g., by spraying, in drops, as an eyewash, or an ointment. Administration can be provided by the subject or by another person, e.g., a health care provider. The medication can be provided in measured doses or in a dispenser which delivers a metered dose. The medication can also be administered to the interior of the eye, and can be introduced by a needle or other delivery device which can introduce it to a selected area or structure. Ocular treatment is particularly desirable for treating inflammation of the eye or nearby tissue.

Any of the iRNA agents described herein can be administered directly to the skin. For example, the medication can be applied topically or delivered in a layer of the skin, e.g., by the use of a microneedle or a battery of microneedles which penetrate into the skin, but preferably not into the underlying muscle tissue. Administration of the iRNA agent composition can be topical. Topical applications can, for example, deliver the composition to the dermis or epidermis of a subject. Topical administration can be in the form of transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids or powders. A composition for topical administration can be formulated as a liposome, micelle, emulsion, or other lipophilic

molecular assembly. The transdermal administration can be applied with at least one penetration enhancer, such as iontophoresis, phonophoresis, and sonophoresis.

Any of the iRNA agents described herein can be administered to the pulmonary system. Pulmonary administration can be achieved by inhalation or by the introduction of a delivery device into the pulmonary system, *e.g.*, by introducing a delivery device which can dispense the medication. A preferred method of pulmonary delivery is by inhalation. The medication can be provided in a dispenser which delivers the medication, *e.g.*, wet or dry, in a form sufficiently small such that it can be inhaled. The device can deliver a metered dose of medication. The subject, or another person, can administer the medication.

Pulmonary delivery is effective not only for disorders which directly affect pulmonary tissue, but also for disorders which affect other tissue.

iRNA agents can be formulated as a liquid or nonliquid, *e.g.*, a powder, crystal, or aerosol for pulmonary delivery.

Any of the iRNA agents described herein can be administered nasally. Nasal administration can be achieved by introduction of a delivery device into the nose, *e.g.*, by introducing a delivery device which can dispense the medication. Methods of nasal delivery include spray, aerosol, liquid, *e.g.*, by drops, or by topical administration to a surface of the nasal cavity. The medication can be provided in a dispenser with delivery of the medication, *e.g.*, wet or dry, in a form sufficiently small such that it can be inhaled. The device can deliver a metered dose of medication. The subject, or another person, can administer the medication.

Nasal delivery is effective not only for disorders which directly affect nasal tissue, but also for disorders which affect other tissue

iRNA agents can be formulated as a liquid or nonliquid, *e.g.*, a powder, crystal, or for nasal delivery.

An iRNA agent can be packaged in a viral natural capsid or in a chemically or enzymatically produced artificial capsid or structure derived therefrom.

The dosage of a pharmaceutical composition including a iRNA agent can be administered in order to alleviate the symptoms of a disease state, *e.g.*, cancer or a cardiovascular disease. A

subject can be treated with the pharmaceutical composition by any of the methods mentioned above.

Gene expression in a subject can be modulated by administering a pharmaceutical composition including an iRNA agent.

5   A subject can be treated by administering a defined amount of an iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, (e.g., a precursor, e.g., a larger iRNA agent which can be processed into a sRNA agent) composition that is in a powdered form, e.g., a collection of microparticles, such as crystalline particles. The composition can include a plurality of iRNA agents, e.g., specific for one or more different endogenous target RNAs. The method can include
10   other features described herein.

A subject can be treated by administering a defined amount of an iRNA agent composition that is prepared by a method that includes spray-drying, i.e. atomizing a liquid solution, emulsion, or suspension, immediately exposing the droplets to a drying gas, and collecting the resulting porous powder particles. The composition can include a plurality of
15   iRNA agents, e.g., specific for one or more different endogenous target RNAs. The method can include other features described herein.

The iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, (e.g., a precursor, e.g., a larger iRNA agent which can be processed into a sRNA agent, or a DNA which encodes an iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, or precursor thereof), can be
20   provided in a powdered, crystallized or other finely divided form, with or without a carrier, e.g., a micro- or nano-particle suitable for inhalation or other pulmonary delivery. This can include providing an aerosol preparation, e.g., an aerosolized spray-dried composition. The aerosol composition can be provided in and/or dispensed by a metered dose delivery device.

The subject can be treated for a condition treatable by inhalation, e.g., by aerosolizing a
25   spray-dried iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, (e.g., a precursor, e.g., a larger iRNA agent which can be processed into a sRNA agent, or a DNA which encodes an iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, or precursor thereof) composition and inhaling the aerosolized composition. The iRNA agent can be an sRNA. The composition can include a plurality of iRNA agents, e.g., specific for one or more different
30   endogenous target RNAs. The method can include other features described herein.

A subject can be treated by, for example, administering a composition including an effective/defined amount of an iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, (e.g., a precursor, e.g., a larger iRNA agent which can be processed into a sRNA agent, or a DNA which encodes an iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, or precursor thereof), wherein the composition is prepared by a method that includes spray-drying, lyophilization, vacuum drying, evaporation, fluid bed drying, or a combination of these techniques

In another aspect, the invention features a method that includes: evaluating a parameter related to the abundance of a transcript in a cell of a subject; comparing the evaluated parameter to a reference value; and if the evaluated parameter has a preselected relationship to the reference value (e.g., it is greater), administering a iRNA agent (or a precursor, e.g., a larger iRNA agent which can be processed into a sRNA agent, or a DNA which encodes a iRNA agent or precursor thereof) to the subject. In one embodiment, the iRNA agent includes a sequence that is complementary to the evaluated transcript. For example, the parameter can be a direct measure of transcript levels, a measure of a protein level, a disease or disorder symptom or characterization (e.g., rate of cell proliferation and/or tumor mass, viral load).

In another aspect, the invention features a method that includes: administering a first amount of a composition that comprises an iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, (e.g., a precursor, e.g., a larger iRNA agent which can be processed into a sRNA agent, or a DNA which encodes an iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, or precursor thereof) to a subject, wherein the iRNA agent includes a strand substantially complementary to a target nucleic acid; evaluating an activity associated with a protein encoded by the target nucleic acid; wherein the evaluation is used to determine if a second amount should be administered. In a preferred embodiment the method includes administering a second amount of the composition, wherein the timing of administration or dosage of the second amount is a function of the evaluating. The method can include other features described herein.

In another aspect, the invention features a method of administering a source of a double-stranded iRNA agent (ds iRNA agent) to a subject. The method includes administering or implanting a source of a ds iRNA agent, e.g., a sRNA agent, that (a) includes a double-stranded region that is 19-25 nucleotides long, preferably 21-23 nucleotides, (b) is complementary to a target RNA (e.g., an endogenous RNA or a pathogen RNA), and, optionally, (c) includes at least one 3' overhang 1-5 nt long. In one embodiment, the source releases ds iRNA agent over time,

e.g. the source is a controlled or a slow release source, *e.g.*, a microparticle that gradually releases the ds iRNA agent. In another embodiment, the source is a pump, *e.g.*, a pump that includes a sensor or a pump that can release one or more unit doses.

In one aspect, the invention features a pharmaceutical composition that includes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or sRNA agent, (*e.g.*, a precursor, *e.g.*, a larger iRNA agent which can be processed into a sRNA agent, or a DNA which encodes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or sRNA agent, or precursor thereof) including a nucleotide sequence complementary to a target RNA, *e.g.*, substantially and/or exactly complementary. The target RNA can be a transcript of an endogenous human gene. In one embodiment, the iRNA agent (a) is 19-25 nucleotides long, preferably 21-23 nucleotides, (b) is complementary to an endogenous target RNA, and, optionally, (c) includes at least one 3' overhang 1-5 nt long. In one embodiment, the pharmaceutical composition can be an emulsion, microemulsion, cream, jelly, or liposome.

In one example the pharmaceutical composition includes an iRNA agent mixed with a topical delivery agent. The topical delivery agent can be a plurality of microscopic vesicles. The microscopic vesicles can be liposomes. In a preferred embodiment the liposomes are cationic liposomes.

In another aspect, the pharmaceutical composition includes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or sRNA agent (*e.g.*, a precursor, *e.g.*, a larger iRNA agent which can be processed into a sRNA agent, or a DNA which encodes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or sRNA agent, or precursor thereof) admixed with a topical penetration enhancer. In one embodiment, the topical penetration enhancer is a fatty acid. The fatty acid can be arachidonic acid, oleic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a C_{1-10} alkyl ester, monoglyceride, diglyceride or pharmaceutically acceptable salt thereof.

In another embodiment, the topical penetration enhancer is a bile salt. The bile salt can be cholic acid, dehydrocholic acid, deoxycholic acid, glucholic acid, glycholic acid, glycodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, chenodeoxycholic acid, ursodeoxycholic acid, sodium tauro-24,25-dihydro-fusidate, sodium glycodihydrofusidate, polyoxyethylene-9-lauryl ether or a pharmaceutically acceptable salt thereof.

In another embodiment, the penetration enhancer is a chelating agent. The chelating agent can be EDTA, citric acid, a salicyclate, a N-acyl derivative of collagen, laureth-9, an N-amino acyl derivative of a beta-diketone or a mixture thereof.

In another embodiment, the penetration enhancer is a surfactant, *e.g.*, an ionic or nonionic surfactant. The surfactant can be sodium lauryl sulfate, polyoxyethylene-9-lauryl ether, polyoxyethylene-20-cetyl ether, a perfluorchemical emulsion or mixture thereof.

In another embodiment, the penetration enhancer can be selected from a group consisting of unsaturated cyclic ureas, 1-alkyl-alkones, 1-alkenylazacyclo-alakanones, steroidal anti-inflammatory agents and mixtures thereof. In yet another embodiment the penetration enhancer can be a glycol, a pyrrol, an azone, or a terpenes.

In one aspect, the invention features a pharmaceutical composition including an iRNA agent, *e.g.*, a double-stranded iRNA agent, or sRNA agent, (*e.g.*, a precursor, *e.g.*, a larger iRNA agent which can be processed into a sRNA agent, or a DNA which encodes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or sRNA agent, or precursor thereof) in a form suitable for oral delivery. In one embodiment, oral delivery can be used to deliver an iRNA agent composition to a cell or a region of the gastro-intestinal tract, *e.g.*, small intestine, colon (*e.g.*, to treat a colon cancer), and so forth. The oral delivery form can be tablets, capsules or gel capsules. In one embodiment, the iRNA agent of the pharmaceutical composition modulates expression of a cellular adhesion protein, modulates a rate of cellular proliferation, or has biological activity against eukaryotic pathogens or retroviruses. In another embodiment, the pharmaceutical composition includes an enteric material that substantially prevents dissolution of the tablets, capsules or gel capsules in a mammalian stomach. In a preferred embodiment the enteric material is a coating. The coating can be acetate phthalate, propylene glycol, sorbitan monoleate, cellulose acetate trimellitate, hydroxy propyl methylcellulose phthalate or cellulose acetate phthalate.

In another embodiment, the oral dosage form of the pharmaceutical composition includes a penetration enhancer. The penetration enhancer can be a bile salt or a fatty acid. The bile salt can be ursodeoxycholic acid, chenodeoxycholic acid, and salts thereof. The fatty acid can be capric acid, lauric acid, and salts thereof.

In another embodiment, the oral dosage form of the pharmaceutical composition includes an excipient. In one example the excipient is polyethyleneglycol. In another example the excipient is precirol.

In another embodiment, the oral dosage form of the pharmaceutical composition includes a plasticizer. The plasticizer can be diethyl phthalate, triacetin dibutyl sebacate, dibutyl phthalate or triethyl citrate.

In one aspect, the invention features a pharmaceutical composition including an iRNA agent and a delivery vehicle. In one embodiment, the iRNA agent is (a) is 19-25 nucleotides long, preferably 21-23 nucleotides, (b) is complementary to an endogenous target RNA, and, optionally, (c) includes at least one 3' overhang 1-5 nucleotides long.

In one embodiment, the delivery vehicle can deliver an iRNA agent, *e.g.*, a double-stranded iRNA agent, or sRNA agent, (*e.g.*, a precursor, *e.g.*, a larger iRNA agent which can be processed into a sRNA agent, or a DNA which encodes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or sRNA agent, or precursor thereof) to a cell by a topical route of administration. The delivery vehicle can be microscopic vesicles. In one example the microscopic vesicles are liposomes. In a preferred embodiment the liposomes are cationic liposomes. In another example the microscopic vesicles are micelles.In one aspect, the invention features a pharmaceutical composition including an iRNA agent, *e.g.*, a double-stranded iRNA agent, or sRNA agent, (*e.g.*, a precursor, *e.g.*, a larger iRNA agent which can be processed into a sRNA agent, or a DNA which encodes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or sRNA agent, or precursor thereof) in an injectable dosage form. In one embodiment, the injectable dosage form of the pharmaceutical composition includes sterile aqueous solutions or dispersions and sterile powders. In a preferred embodiment the sterile solution can include a diluent such as water; saline solution; fixed oils, polyethylene glycols, glycerin, or propylene glycol.

In one aspect, the invention features a pharmaceutical composition including an iRNA agent, *e.g.*, a double-stranded iRNA agent, or sRNA agent, (*e.g.*, a precursor, *e.g.*, a larger iRNA agent which can be processed into a sRNA agent, or a DNA which encodes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or sRNA agent, or precursor thereof) in oral dosage form. In one embodiment, the oral dosage form is selected from the group consisting of tablets, capsules and gel capsules. In another embodiment, the pharmaceutical composition includes an enteric material that substantially prevents dissolution of the tablets, capsules or gel capsules in a mammalian stomach. In a preferred embodiment the enteric material is a coating. The coating

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can be acetate phthalate, propylene glycol, sorbitan monoleate, cellulose acetate trimellitate, hydroxy propyl methyl cellulose phthalate or cellulose acetate phthalate. In one embodiment, the oral dosage form of the pharmaceutical composition includes a penetration enhancer, e.g., a penetration enhancer described herein.

5   In one aspect, the invention features a pharmaceutical composition including an iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, (e.g., a precursor, e.g., a larger iRNA agent which can be processed into a sRNA agent, or a DNA which encodes an iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, or precursor thereof) in a rectal dosage form. In one embodiment, the rectal dosage form is an enema. In another embodiment, the rectal dosage
10   form is a suppository.

        In one aspect, the invention features a pharmaceutical composition including an iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, (e.g., a precursor, e.g., a larger iRNA agent which can be processed into a sRNA agent, or a DNA which encodes an iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, or precursor thereof) in a vaginal dosage form.
15   In one embodiment, the vaginal dosage form is a suppository. In another embodiment, the vaginal dosage form is a foam, cream, or gel.

        In one aspect, the invention features a pharmaceutical composition including an iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, (e.g., a precursor, e.g., a larger iRNA agent which can be processed into a sRNA agent, or a DNA which encodes an iRNA agent, e.g.,
20   a double-stranded iRNA agent, or sRNA agent, or precursor thereof) in a pulmonary or nasal dosage form. In one embodiment, the iRNA agent is incorporated into a particle, e.g., a macroparticle, e.g., a microsphere. The particle can be produced by spray drying, lyophilization, evaporation, fluid bed drying, vacuum drying, or a combination thereof. The microsphere can be formulated as a suspension, a powder, or an implantable solid.

25   In one aspect, the invention features a spray-dried iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, (e.g., a precursor, e.g., a larger iRNA agent which can be processed into a sRNA agent, or a DNA which encodes an iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, or precursor thereof) composition suitable for inhalation by a subject, including: (a) a therapeutically effective amount of a iRNA agent suitable for treating a condition
30   in the subject by inhalation; (b) a pharmaceutically acceptable excipient selected from the group consisting of carbohydrates and amino acids; and (c) optionally, a dispersibility-enhancing amount of a physiologically-acceptable, water-soluble polypeptide.

In one embodiment, the excipient is a carbohydrate. The carbohydrate can be selected from the group consisting of monosaccharides, disaccharides, trisaccharides, and polysaccharides. In a preferred embodiment the carbohydrate is a monosaccharide selected from the group consisting of dextrose, galactose, mannitol, D-mannose, sorbitol, and sorbose. In another preferred embodiment the carbohydrate is a disaccharide selected from the group consisting of lactose, maltose, sucrose, and trehalose.

In another embodiment, the excipient is an amino acid. In one embodiment, the amino acid is a hydrophobic amino acid. In a preferred embodiment the hydrophobic amino acid is selected from the group consisting of alanine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan, and valine. In yet another embodiment the amino acid is a polar amino acid. In a preferred embodiment the amino acid is selected from the group consisting of arginine, histidine, lysine, cysteine, glycine, glutamine, serine, threonine, tyrosine, aspartic acid and glutamic acid.

In one embodiment, the dispersibility-enhancing polypeptide is selected from the group consisting of human serum albumin, α-lactalbumin, trypsinogen, and polyalanine.

In one embodiment, the spray-dried iRNA agent composition includes particles having a mass median diameter (MMD) of less than 10 microns. In another embodiment, the spray-dried iRNA agent composition includes particles having a mass median diameter of less than 5 microns. In yet another embodiment the spray-dried iRNA agent composition includes particles having a mass median aerodynamic diameter (MMAD) of less than 5 microns.

In certain other aspects, the invention provides kits that include a suitable container containing a pharmaceutical formulation of an iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, (e.g., a precursor, e.g., a larger iRNA agent which can be processed into a sRNA agent, or a DNA which encodes an iRNA agent, e.g., a double-stranded iRNA agent, or sRNA agent, or precursor thereof). In certain embodiments the individual components of the pharmaceutical formulation may be provided in one container. Alternatively, it may be desirable to provide the components of the pharmaceutical formulation separately in two or more containers, e.g., one container for an iRNA agent preparation, and at least another for a carrier compound. The kit may be packaged in a number of different configurations such as one or more containers in a single box. The different components can be combined, e.g., according to instructions provided with the kit. The components can be combined according to a method

described herein, *e.g.*, to prepare and administer a pharmaceutical composition. The kit can also include a delivery device.

In another aspect, the invention features a device, *e.g.*, an implantable device, wherein the device can dispense or administer a composition that includes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or sRNA agent, (*e.g.*, a precursor, *e.g.*, a larger iRNA agent which can be processed into a sRNA agent, or a DNA which encodes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or sRNA agent, or precursor thereof), *e.g.*, a iRNA agent that silences an endogenous transcript. In one embodiment, the device is coated with the composition. In another embodiment the iRNA agent is disposed within the device. In another embodiment, the device includes a mechanism to dispense a unit dose of the composition. In other embodiments the device releases the composition continuously, *e.g.*, by diffusion. Exemplary devices include stents, catheters, pumps, artificial organs or organ components (*e.g.*, artificial heart, a heart valve, etc.), and sutures.

As used herein, the term "crystalline" describes a solid having the structure or characteristics of a crystal, *i.e.*, particles of three-dimensional structure in which the plane faces intersect at definite angles and in which there is a regular internal structure. The compositions of the invention may have different crystalline forms. Crystalline forms can be prepared by a variety of methods, including, for example, spray drying.

The invention is further illustrated by the following examples, which should not be construed as further limiting.

EXAMPLES

Example 1: Inhibition of endogenous ApoM gene expression in mice

Apolipoprotein M (ApoM) is a human apolipoprotein predominantly present in high-density lipoprotein (HDL) in plasma. ApoM is reported to be expressed exclusively in liver and in kidney (Xu N *et al.*, Biochem J Biol Chem 1999 Oct 29;274(44):31286-90). Mouse ApoM is a 21kD membrane associated protein, and, in serum, the protein is associated with HDL particles. ApoM gene expression is regulated by the transcription factor hepatocyte nuclear factor 1 alpha (Hnf-1α), as Hnf-1α$^{-/-}$ mice are ApoM deficient. In humans, mutations in the HNF-1 alpha gene represent a common cause of maturity-onset diabetes of the young (MODY).

A variety of test iRNAs were synthesized to target the mouse ApoM gene. This gene was chosen in part because of its high expression levels and exclusive activity in the liver and kidney.

Three different classes of dsRNA agents were synthesized, each class having different modifications and features at the 5′ and 3′ ends, see Table 7.

Table 7

```
Targeted ORF's

     5  The 23mer: AAGTTTGGGCAGCTCTGCTCT  (SEQ ID NO:26)


    19  The 23mer: AAGTGGACATACCGATTGACT  (SEQ ID NO:27)


    25  The 23mer: AACTCAGAACTGAAGGGCGCC  (SEQ ID NO:28)


    27  The 23mer: AAGGGCGCCCAGACATGAAAA  (SEQ ID NO:29)


3'-UTR

    42: AAGATAGGAGCCCAGCTTCGA   (SEQ ID NO:30)
```

<u>Class I</u>

21-nt iRNAs, t, deoxythymidine; p, phosphate

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pGUUUGGGCAGCUCUGCUCUtt  (SEQ ID NO:31) #1

pAGAGCAGAGCUGCCCAAACtt  (SEQ ID NO:32)
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pGUGGACAUACCGAUUGACUtt (SEQ ID NO:33) #2

pAGUCAAUCGGUAUGUCCACtt (SEQ ID NO:34)

pCUCAGAACUGAAGGGCGCCtt (SEQ ID NO:35) #3

pGGCGCCCUUCAGUUCUGAGtt (SEQ ID NO:36)

pGAUAGGAGCCCAGCUUCGAtt (SEQ ID NO:37) #4

pUCGAAGCUGGGCUCCUAUCtt (SEQ ID NO:38)

Class II

21-nt iRNAs, t, deoxythymidine; p, phosphate; ps, thiophosphate

pGUUUGGGCAGCUCUGCUCpsUpstpst (SEQ ID NO:39) #11

pAGAGCAGAGCUGCCCAAApsCpstpst   (SEQ ID NO:40)

pGUGGACAUACCGAUUGACpsUpstpst (SEQ ID NO:41) #13

pAGUCAAUCGGUAUGUCCApsCpstpst   (SEQ ID NO:42)

pCUCAGAACUGAAGGGCGCpsCpstpst (SEQ ID NO:43) #15

pGGCGCCCUUCAGUUCUGApsGpstpst (SEQ ID NO:44)

pGAUAGGAGCCCAGCUUCGpsApstpst (SEQ ID NO:45) #17

pUCGAAGCUGGGCUCCUAUpsCpstpst   (SEQ ID NO:46)

Class III

23-nt antisense, 21-nt sense, blunt-ended 5'-as


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GUUUGGGCAGCUCUGCUCUCU   (SEQ ID NO:47) #19

AGAGAGCAGAGCUGCCCAAACUU      (SEQ ID NO:48)


GUGGACAUACCGAUUGACUGA   (SEQ ID NO:49) #21

UCAGUCAAUCGGUAUGUCCACUU      (SEQ ID NO:50)


CUCAGAACUGAAGGGCGCCCA   (SEQ ID NO:51) #23

PUGGGCGCCCUUCAGUUCUGAGUU      (SEQ ID NO:52)


GAUAGGAGCCCAGCUUCGAGU   (SEQ ID NO:53) #25

ACUCGAAGCUGGGCUCCUAUCUU      (SEQ ID NO:54)
```

Class I dsRNAs consisted of 21 nucleotide paired sense and antisense strands. The sense and antisense strands were each phosphorylated at their 5' ends. The double stranded region was 19 nucleotides long and consisted of ribonucleotides. The 3' end of each strand created a two nucleotide overhang consisting of two deoxyribonucleotide thymidines. See constructs #1-4 in Table 7.

Class II dsRNAs were also 21 nucleotides long, with a 19 nucleotide double strand region. The sense and antisense strands were each phosphorylated at their 5' ends. The three 3' terminal nucleotides of the sense and antisense strands were phosphorothioate deoxyribonucleotides, and the two terminal phosphorothioate thymidines were unpaired, creating a 3' overhang region at each end of the iRNA molecule. See constructs 11, 13, 15, and 17 in Table 7.

Class III dsRNAs included a 23 ribonucleotide antisense strand and a 21 ribonucleotide sense strand, to form a construct having a blunt 5' and a 3' overhang region. See constructs 19, 21, 23, and 25 in Table 7.

Within each of the three classes of iRNAs, the four dsRNA molecules were designed to target four different regions of the ApoM transcript. dsRNAs 1, 11, and 19 targeted the 5' end of the open reading frame (ORF). dsRNAs 2, 13, and 21, and 3, 15, and 23, targeted two internal regions (one 5' proximal and one 3' proximal) of the ORF, and the 4, 17, and 25 iRNA constructs targeted to a region of the 3' untranslated sequence (3' UTS) of the ApoM mRNA. This is summarized in Table 8.

Table 8. iRNA molecules targeted to mouse ApoM

	iRNA targeted to 5' end of ORF	iRNA targeted to middle ORF (5' proximal)	iRNA targeted to middle ORF (3' proximal)	iRNA targeted to 3'UTS
Class I	1	2	3	4
Class II	11	13	15	17
Class III	19	21	23	25

CD1 mice (6-8 weeks old, ~35g) were administered one of the test iRNAs in PBS solution. Two hundred micrograms of iRNA in a volume of solution equal to 10% body weight (~5.7mg iRNA/kg mouse) was administered by the method of high pressure tail vein injection, over a 10-20 sec. time interval. After a 24h recovery period, a second injection was performed using the same dose and mode of administration as the first injection, and following another 24h, a third and final injection was administered, also using the same dose and mode of administration. After a final 24h recovery, the mouse was sacrificed, serum was collected and the liver and kidney harvested to assay for an affect on ApoM gene expression. Expression was

monitored by quantitative RT-PCR and Western blot analyses. This experiment was repeated for each of the iRNAs listed in table 7.

Class I iRNAs did not alter ApoM RNA levels in mice, as indicated by quantitative RT-PCR (FIGs. 7A and 7B). This is in contrast to the effect of these iRNAs in cultured HepG2 cells (FIG. 8). Cells cotransfected with a plasmid expressing exogenous ApoM RNA under a CMV promoter and a class I iRNA demonstrated a 25% or greater reduction in ApoM RNA concentrations as compared to control transfections. The iRNA molecules 1, 2 and 3 each caused a 75% decrease in exogenous ApoM mRNA levels.

Class II iRNAs reduced liver and kidney ApoM mRNA levels by ~30-85% (FIGs. 9A and 9B). The iRNA molecule "13" elicited the most dramatic reduction in mRNA levels; quantitative RT-PCR indicated a decrease of about 85% in liver tissue. Serum ApoM protein levels were also reduced as was evidenced by Western blot analysis. The iRNAs 11, 13 and 15, reduced protein levels by about 50%, while iRNA 17 had the mildest effect, reducing levels only by ~15-20% (FIGs. 10A and 10B).

Class III iRNAs (constructs 19, 21, and 23) reduced serum ApoM levels by ~40-50% (FIGs. 11A and 11B).

To determine the effect of dosage on iRNA mediated ApoM inhibition, the experiment described above was repeated with three injections of 50 μg iRNA "11" (~1.4 mg iRNA/kg mouse). This lower dosage of iRNA resulted in a reduction of serum ApoM level of about 50% (FIGs. 12A and 12B). This is compared with the reduction seen with the 200 μg injections, which reduced serum levels by 25-45%. These results indicated the lower dosage amounts of iRNAs were effective.

In an effort to increase iRNA uptake by cells, iRNAs were precomplexed with lipofectamine prior to tail vein injections. ApoM protein levels were about 50% of wildtype levels in mice injected with iRNA "11" when the molecules were preincubated with lipofectamine; ApoM levels were also about 50% of wildtype when mice were injected with iRNA "11" that was not precomplexed with lipofectamine (FIGs. 12A and 12B).

These experiments revealed that modified iRNAs can greatly influence RNAi-mediated gene silencing. As demonstrated herein, modifications including phosphorothioate nucleotides are particularly effective at decreasing target protein levels.

Example 2: Design of iRNA agents to target SGLT2

     The major reabsorptive mechanism for D-glucose in the kidney involves a low affinity, high capacity Na+/glucose cotransporter, sodium-glucose cotransporter 2 (OMIM 182381) or SGLT2. SGLT2 and 3 are much more restrictive than SGLT1 in that they prefer D-glucose to D-galactose or 3-O-methyl-D-glucose. Wright (*Am. J. Physiol. Renal Physiol.* 280:F10-18, 2001) and Kanai *et al.* (*J. Clin. Invest.* 93:397-404, 1994) demonstrated an extremely high level of SGLT2 message in proximal tubule S1 segments. This level of expression was also evident on Northern blots and likely confers the high capacity of this glucose transport system.

     Blocking the increase in glucose reabsorption by the proximal tubule in patients with early-stage renal disease may prevent progression to renal failure. For example, phlorizin, a small molecule known to block Na$^+$/glucose cotransport (see structure below), reduced the single nephron glomerular filtration rate. The glomerular filtration rate is increased significantly in patients with early diabetes mellitus as compared to normal volunteers. Down-regulation of SGLT2 protein production can lead to significant therapeutic improvement.
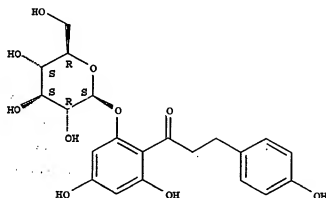
Phlorizin

     Five siRNA oligonucleotides that target the SGLT2 mRNA sequence were selected (see FIG. 6). The cDNA for SGLT2 was sequenced by Wells *et al.* (*Genomics* 17:787-789, 1993). This sequence includes coding region and some UTR sequence. The iRNA target sequences were selected using Dharmacon selection tools (Dharmacon, Inc., Lafayette, CO) and are shown in Table 1. The Dharmacon selection algorithm allows the user to select certain parameters, including GC content, starting sequence, and mRNA region (*i.e.*, only cDNAs). It also allows one to exclude certain sequences including nucleotide triplets. Oligonucleotides are selected

based on the rules developed by Elbashir *et al.* (*Methods* 26:199-213, 2002). The biggest

advantage of the Dharmacon selection algorithm, in contrast to those offered by Qiagen, Inc.

(Valencia, CA), Ambion, Inc. (Austin, TX), and Sfold (Ding and Lawrence, Nucleic Acids Res.

29:1034-1046, 2001) is that the chosen sequences are automatically BLASTed against sequences

5   in GenBank for the selected organism. The automatic BLAST step saves considerable time and

ensures that the oligonucleotides selected do not have significant homology to another known

gene.

OTHER EMBODIMENTS

While this invention has been particularly shown and described with reference to

10   preferred embodiments thereof, it will be understood by those skilled in the art that various

changes in form and details may be made therein without departing from the scope of the

invention encompassed by the appended claims.

WHAT IS CLAIMED IS:

1. An iRNA agent comprising a sense sequence and an antisense sequence, wherein the a the iRNA agent comprises a stabilizing modification, and wherein the antisense sequence targets an RNA expressed in kidney.

5   2. The iRNA agent of claim 1, wherein the stabilizing modification is a cationic group.

3. The iRNA agent of claim 1, wherein the stabilizing modification is a 2'-O-alkyl amine, 2'-O-alkoxyalkyl amine, a polyamine, a C5-cationic modified pyrimidine, a cationic peptide, guanidinium group, amidininium group or a cationic amino acid.

4. The iRNA agent of claim 1, further comprising a modification that alters the
10   distribution of the iRNA agent in favor of the kidney.

5. The iRNA agent of claim 4, wherein the modification is one or more of the following: a cationic group; a carbohydrate sugar; an enzyme or enzyme substrate; folic acid; a protein; a water soluble molecule; an RGD peptide, or any derivative or analog thereof; a synthetic molecule capable of targeting alpha v-beta 3 integrin..

15   6. The iRNA agent of claim 5, wherein the iRNA agent binds alpha v-beta 3 integrin.

7. The iRNA agent of claim 1, wherein the sense sequence comprises one or more asymmetrical 2'O alkyl amine modifications.

8. The iRNA agent of claim 1, wherein the antisense sequence comprises one or more asymmetrical phosphorothioate modifications.

20   9. The iRNA agent of claim 7, wherein at least one of said 2'-O-alkyl amine modifications is a 2'-O-propylamine or 2'-O-(dimethylaminooxyethyl) modification.

10. The iRNA agent of claim 1, having a nucleobase modification.

11. The iRNA agent of claim 10, wherein said nucleobase modification is selected from a C-5 pyrimidine, an N-2 purine, an N-7 purine, and an N-6 purine modification.

25   12. The iRNA agent of claim 1 or 11 having a modification which includes a cationic group or a Zwitterionic group.

13. The iRNA agent of claim 12, wherein said modification is at a terminus.

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14. The iRNA agent of claim 1, modified to include one or more of a cationic group or a Zwitterionic group at a 2' position of a sugar, a 3' position of a sugar, the C-5 of a pyrimidine, the N-2 of a purine, the N-7 of a purine, or the N-6 of a purine.

15. The iRNA agent of claim 1, modified to include a guanidinium group at a 2' position of a sugar, a 3' position of a sugar, the C-5 of a pyrimidine, the N-2 of a purine, the N-7 of a purine, or the N-6 of a purine.

16. The iRNA agent of claim 1, wherein the sense sequence has at least 2 asymmetrical 2'-O alkyl amine modifications.

17. The iRNA agent of claim 1, wherein the sense sequence has at least 4 asymmetrical 2'-O alkyl amine modifications.

18. The iRNA agent of claim 17, wherein the asymmetrical modifications are 2'-OMe modifications.

19. The iRNA agent of claim 1, wherein the sense sequence has at least 6 asymmetrical 2'-O alkyl amine modifications.

20. The iRNA agent of claim 19, wherein the asymmetrical modifications are 2'-OMe modifications.

21. The iRNA agent of claim 1, wherein the sense sequence has at least 8 asymmetrical 2'-O alkyl amine modifications.

22. The iRNA agent of claim 21, wherein the asymmetrical modifications are 2'-OMe modifications.

23. The iRNA agent of claim 1, wherein all of the subunits of the sense sequence have an asymmetrical 2'-O alkyl amine modification.

24. The iRNA agent of claim 23, wherein the asymmetrical modification is a 2'-OMe modification.

25. The iRNA agent of claim 1, wherein the antisense sequence has at least 2 asymmetrical phosphorothioate modifications.

26. The iRNA agent of claim 1, wherein the antisense sequence has at least 4 asymmetrical phosphorothioate modifications.

27. The iRNA agent of claim 1, wherein the antisense sequence has at least 6 asymmetrical phosphorothioate modifications.

28. The iRNA agent of claim 1, wherein the antisense sequence has at least 8 asymmetrical phosphorothioate modifications.

29. A method for treating a subject having or at risk for having a disorder of the kidney, the method comprising administering to a subject an iRNA agent which targets an RNA expressed in the kidney, wherein said agent is modified to alter its distribution in favor of the kidney.

30. The method of claim 29, wherein said iRNA agent is conjugated with one or more of the following: a cationic group, a sugar, an enzyme or enzyme substrate, folic acid, a protein, a water soluble molecule, an RGD peptide, or any derivative or analog thereof.

31. The method of claim 29, wherein the iRNA agent is at least 21 nucleotides in length, and the duplex region of the iRNA is about 19 nucleotides in length.

32. The method of claim 29, wherein the iRNA agent comprises a sense sequence and an antisense sequence, wherein the sense sequence has one or more asymmetrical 2'-O alkyl amine modifications and the antisense sequence has one or more asymmetrical phosphorothioate modifications.

33. The method of claim 32, wherein at least one of said 2'-O-alkyl amine modifications is a 2'-OMe modification.

34. The method of claim 32, wherein the sense sequence has at least 2 asymmetrical 2'-O alkyl amine modifications.

35. The method of claim 34, wherein the asymmetrical modifications are 2'-OMe modifications.

36. The method of claim 32, wherein the sense sequence has at least 4 asymmetrical 2'-O alkyl amine modifications.

37. The method of claim 36, wherein the asymmetrical modifications are 2'-OMe modifications.

38. The method of claim 32, wherein the sense sequence has at least 6 asymmetrical 2'-O alkyl amine modifications.

39. The iRNA agent of claim 38, wherein the asymmetrical modifications are 2'-OMe modifications.

40. The method of claim 32, wherein the sense sequence has at least 8 asymmetrical 2'-O alkyl amine modifications.

41. The method of claim 40, wherein the asymmetrical modifications are 2'-OMe modifications.

42. The method of claim 32, wherein all of the subunits of the sense sequence have an asymmetrical 2'-O alkyl amine modification.

43. The method of claim 42, wherein the asymmetrical modifications are 2'-OMe modifications.

44. The method of claim 32, wherein the antisense sequence has at least 2 asymmetrical phosphorothioate modifications.

45. The method of claim 32, wherein the antisense sequence has at least 4 asymmetrical phosphorothioate modifications.

46. The method of claim 32, wherein the antisense sequence has at least 6 asymmetrical phosphorothioate modifications.

47. The method of claim 32, wherein the antisense sequence has at least 8 asymmetrical phosphorothioate modifications.

48. The method of claim 29, wherein the iRNA agent targets a nucleic acid encoding one of the following: a chemokine, a complement factor, a growth factor, a growth factor receptor, a cytokine, or a vasoactive protein.

49. The method of claim 29, wherein the iRNA agent targets one of the following: MCP1, osteopontin, RANTES, TGFbeta, TNFalpha, PDGF, IGF-1, IGF-2, VEGF, IL1alpha,

ET1, angiotensin II, PPARalpha, PPARbeta/delta, PPARgamma, B7-1, B7-2, ICOS, CD40, or CD154.

50. The method of claim 29, wherein the disorder of the kidney is one of the group consisting of ureter obstruction, diabetes, proteinurea, renal carcinoma, Fanconi's syndrome and Bartter's syndrome.

51. The method of claim 29, wherein the iRNA agent is administered to a subject in shock.

52. The method of claim 29, wherein the iRNA agent is administered to a subject before, during, or after a kidney transplant.

53. A preparation of an iRNA agent which targets an RNA expressed in the kidney wherein said agent is modified to alter its distribution in favor of the kidney.

54. The preparation of claim 53, wherein said iRNA agent is conjugated with one or more of the following: a cationic group, a sugar, an enzyme or enzyme substrate, folic acid, a protein, a water soluble molecule, an RGD peptide, or any derivative or analog thereof.

55. The preparation of claim 53, wherein the iRNA agent is at least 21 nucleotides in length, and the duplex region of the iRNA is about 19 nucleotides in length.

56. The preparation of claim 53, wherein the iRNA agent targets a nucleic acid encoding one of the following: a chemokine, a complement factor, a growth factor, a growth factor receptor, a cytokine, or a vasoactive protein.

57. The preparation of claim 53, wherein the iRNA agent targets one of the following: MCP1, osteopontin, RANTES, TGFbeta, TNFalpha, PDGF, IGF-1, IGF-2, VEGF, IL1alpha, ET1, angiotensin II, PPARalpha, PPARbeta/delta, PPARgamma, B7-1, B7-2, ICOS, CD40, or CD154.

58. The preparation of claim 53, wherein said agent is modified to alter its distribution in favor of the proximal tubular cells of the kidney.

59. The preparation of claim 53, wherein said agent is modified to alter its distribution in favor of the glomerulus of the kidney.

60. The preparation of claim 59, wherein said agent is modified to alter its distribution in favor of glomerular epithelial, glomerular endothelial, or mesangial cells of the kidney.

61. A pharamaceutical preparation comprising an iRNA agent which targets an RNA expressed in the kidney wherein said agent is modified to alter its distribution in favor of the kidney.

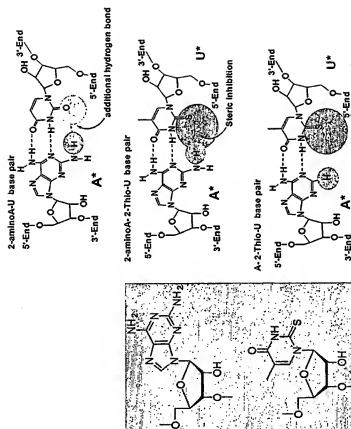62. A composition comprising an iRNA agent and a ligand, wherein the ligand binds a human serum protein.
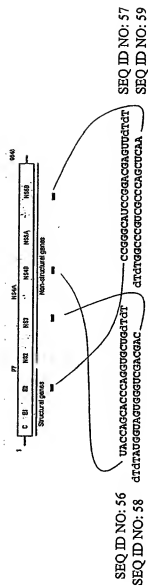
FIG. 1

FIG. 2

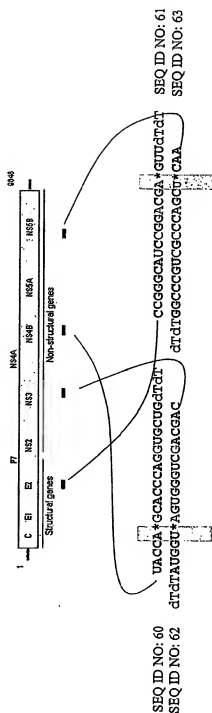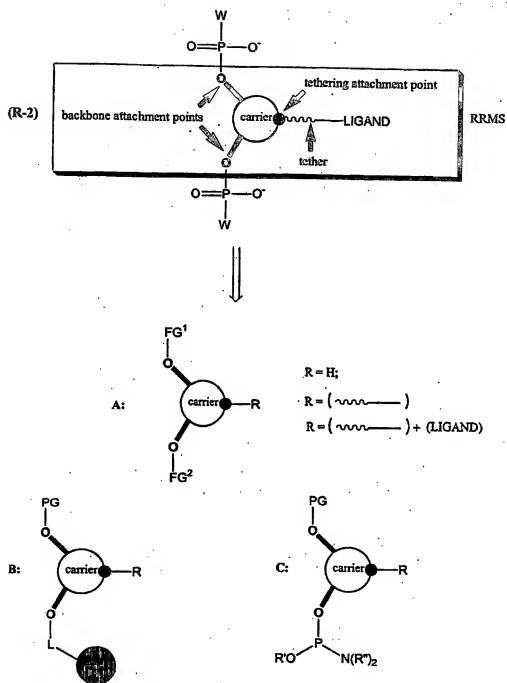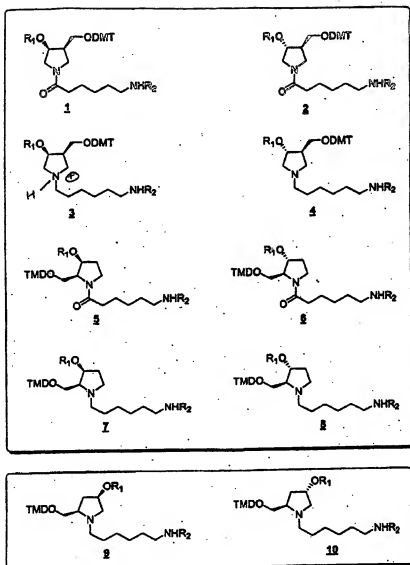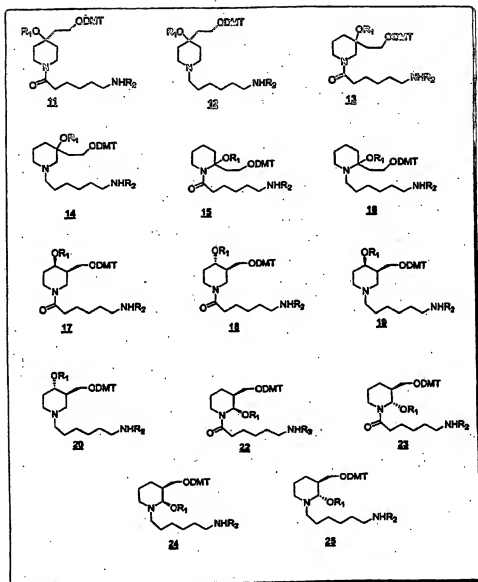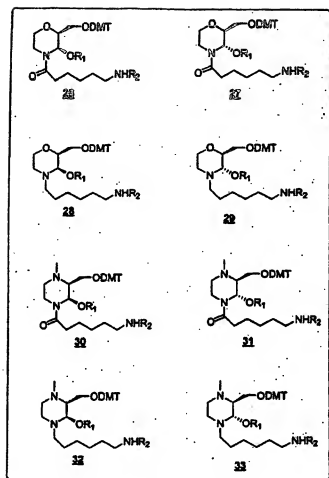FIG. 3
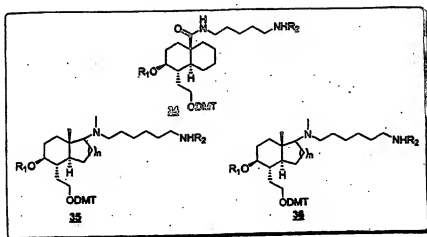
FIG. 4

FIG. 5

FIG. 5 (Cont'd)

FIG. 5 (Cont'd)

FIG. 5 (Cont'd)

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FIG. 6

FIG. 7A
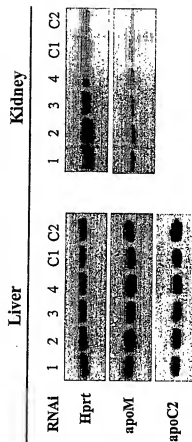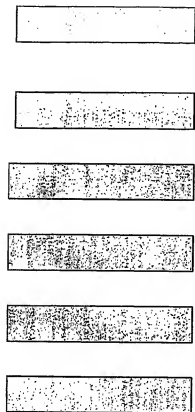
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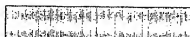
FIG. 8

FIG. 9A
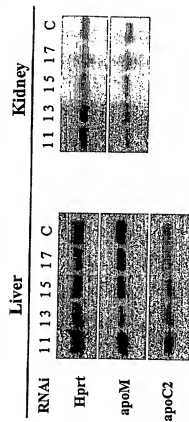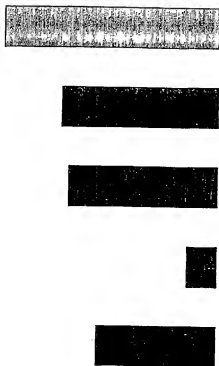
FIG. 9B

FIG. 10A
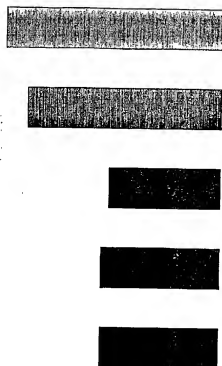
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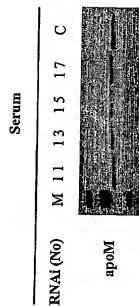
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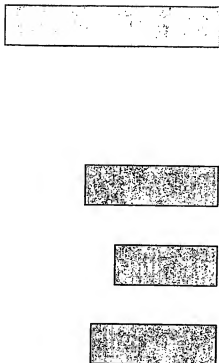
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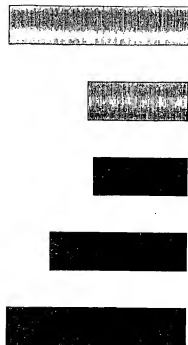
FIG. 12A

FIG. 12B
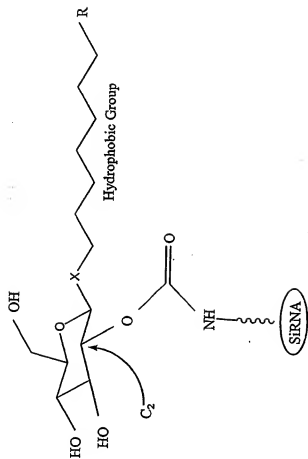
FIG. 13

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1               5                   10


<210> 20
<211> 42
<212> PRT
<213> Artificial Sequence

<220>
<223> Exemplary Cell Permeation Peptides

<400> 20
Arg Arg Arg Pro Arg Pro Pro Tyr Leu Pro Arg Pro Arg Pro Pro Pro
1               5                   10                  15
Phe Phe Pro Pro Arg Leu Pro Pro Arg Ile Pro Pro Gly Phe Pro Pro
            20                  25                  30
Arg Phe Pro Pro Arg Phe Pro Gly Lys Arg
        35                  40

<210> 21
<211> 13
<212> PRT
<213> Artificial Sequence

<220>
<223> Exemplary Cell Permeation Peptides

<400> 21
Ile Leu Pro Trp Lys Trp Pro Trp Trp Pro Trp Arg Arg
1               5                   10

<210> 22
<211> 16
<212> PRT
<213> Artificial Sequence

<220>
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<223> Artificial Sequence

<400> 22
Ala Ala Val Ala Leu Leu Pro Ala Val Leu Leu Ala Leu Leu Ala Pro
1   5   10   15

<210> 23
<211> 11
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetically generated peptide

<400> 23
Ala Ala Leu Leu Pro Val Leu Leu Ala Ala Pro
1   5   10

<210> 24
<211> 13
<212> PRT
<213> Human immunodeficiency virus

<400> 24
Gly Arg Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln
1   5   10

<210> 25
<211> 16
<212> PRT
<213> Drosophila Antennapedia

<400> 25
Arg Gln Ile Lys Ile Trp Phe Gln Asn Arg Arg Met Lys Trp Lys Lys
1   5   10   15

<210> 26
<211> 21
<212> DNA
<213> Mus musculus

<400> 26
aagtttgggc agctctgctc t   21

<210> 27
<211> 21
<212> DNA
<213> Mus musculus

<400> 27
aagtggacat accgattgac t   21

<210> 28
<211> 21
<212> DNA
<213> Mus musculus

<400> 28
aactcagaac tgaagggcgc c   21

<210> 29

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<211> 21
<212> DNA
<213> Mus musculus

<400> 29
aaggcgcccc agacatgaaa a                                    21

<210> 30
<211> 21
<212> DNA
<213> Mus musculus

<400> 30
aagataggag cccagcttcg a                                    21

<210> 31
<211> 21
<212> RNA
<213> Artificial Sequence

<220>
<223> Synthetically generated SiRNA

<221> misc_feature
<222> 20, 21
<223> n = deoxythymidine

<400> 31
guuugggcag cucugcucun n                                    21

<210> 32
<211> 21
<212> RNA
<213> Artificial Sequence

<220>
<223> Synthetically generated SiRNA

<221> misc_feature
<222> 20, 21
<223> n = deoxythymidine

<400> 32
agagcagagc ugcccaaacn n                                    21

<210> 33
<211> 21
<212> RNA
<213> Artificial Sequence

<220>
<223> Synthetically generated SiRNA

<221> misc_feature
<222> 20, 21
<223> n = deoxythymidine

<400> 33
guggacauac cgauugacun n                                    21

<210> 34
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<211> 21
<212> RNA
<213> Artificial Sequence

<220>
<223> Synthetically generated SiRNA

<221> misc_feature
<222> 20, 21
<223> n = deoxythymidine

<400> 34
agucaaucgg uauguccacn n                                              21

<210> 35
<211> 21
<212> RNA
<213> Artificial Sequence

<220>
<223> Synthetically generated SiRNA

<221> misc_feature
<222> 21, 22
<223> n = deoxythymidine

<400> 35
cucagaacug aagggcgccn n                                              21

<210> 36
<211> 21
<212> RNA
<213> Artificial Sequence

<220>
<223> Synthetically generated SiRNA

<221> misc_feature
<222> 20, 21
<223> n = deoxythymidine

<400> 36
ggcgcccuuc aguucugagn n                                              21

<210> 37
<211> 21
<212> RNA
<213> Artificial Sequence

<220>
<223> Synthetically generated SiRNA

<221> misc_feature
<222> 20, 21
<223> n = deoxythymidine

<400> 37
gauaggagcc cagcuucgan n                                              21

<210> 38
<211> 21
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<212> RNA
<213> Artificial Sequence

<220>
<223> Synthetically generated SiRNA

<221> misc_feature
<222> 20, 21
<223> n = deoxythymidine

<400> 38
ucgaagcugg gcuccuaucn n                                               21

<210> 39
<211> 24
<212> RNA
<213> Artificial Sequence

<220>
<223> Synthetically generated SiRNA

<221> misc_feature
<222> 19, 21, 23
<223> n = thiophosphate

<221> misc_feature
<222> 22, 24
<223> n = deoxythymidine

<400> 39
guuugggcag cucugcucnu nnnn                                            24

<210> 40
<211> 24
<212> RNA
<213> Artificial Sequence

<220>
<223> Synthetically generated SiRNA

<221> misc_feature
<222> 19, 21, 23
<223> n = thiophosphate

<221> misc_feature
<222> 22, 24
<223> n = deoxythymidine

<400> 40
agagcagagc ugcccaaanc nnnn                                            24

<210> 41
<211> 24
<212> RNA
<213> Artificial Sequence

<220>
<223> Synthetically generated SiRNA

<221> misc_feature
<222> 19, 21, 23
```

```
<223> n = thiophosphate

<221> misc_feature
<222> 22, 24
<223> n = deoxythymidine

<400> 41
guggacauac cgauugacnu nnnn                                               24

<210> 42
<211> 24
<212> RNA
<213> Artificial Sequence

<220>
<223> Synthetically generated SiRNA

<221> misc_feature
<222> 19, 21, 23
<223> n = thiophosphate

<221> misc_feature
<222> 22, 24
<223> n = deoxythymidine

<400> 42
agucaaucgg uauguccanc nnnn                                               24

<210> 43
<211> 24
<212> RNA
<213> Artificial Sequence

<220>
<223> Synthetically generated SiRNA

<221> misc_feature
<222> 19, 21, 23
<223> n = thiophosphate

<221> misc_feature
<222> 22, 24
<223> n = deoxythymidine

<400> 43
cucagaacug aagggcgcnc nnnn                                               24

<210> 44
<211> 24
<212> RNA
<213> Artificial Sequence

<220>
<223> Synthetically generated SiRNA

<221> misc_feature
<222> 19, 21, 23
<223> n = thiophosphate

<221> misc_feature
<222> 22, 24
```

```
<223> n = deoxythymidine

<400> 44
ggcgcccuuc aguucugang nnnn                                    24

<210> 45
<211> 24
<212> RNA
<213> Artificial Sequence

<220>
<223> Synthetically generated SiRNA

<221> misc_feature
<222> 19, 21, 23
<223> n = thiophosphate

<221> misc_feature
<222> 22, 24
<223> n = deoxythymidine

<400> 45
gauaggagcc cagcuucgna nnnn                                    24

<210> 46
<211> 24
<212> RNA
<213> Artificial Sequence

<220>
<223> Synthetically generated SiRNA

<221> misc_feature
<222> 19, 21, 23
<223> n = thiophosphate

<221> misc_feature
<222> 22, 24
<223> n = deoxythymidine

<400> 46
ucgaagcugg gcuccuaunc nnnn                                    24

<210> 47
<211> 21
<212> RNA
<213> Artificial Sequence

<220>
<223> Synthetically generated SiRNA

<400> 47
guuugggcag cucugcucuc u                                       21

<210> 48
<211> 23
<212> RNA
<213> Artificial Sequence

<220>
<223> Synthetically generated SiRNA
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```
<400> 48
agagagcaga gcugcccaaa cuu                                    23

<210> 49
<211> 21
<212> RNA
<213> Artificial Sequence

<220>
<223> Synthetically generated SiRNA

<400> 49
guggacauac cgauugacug a                                      21

<210> 50
<211> 23
<212> RNA
<213> Artificial Sequence

<220>
<223> Synthetically generated SiRNA

<400> 50
ucagucaauc gguaugucca cuu                                    23

<210> 51
<211> 21
<212> RNA
<213> Artificial Sequence

<220>
<223> Synthetically generated SiRNA

<400> 51
cucagaacug aagggcgccc a                                      21

<210> 52
<211> 23
<212> RNA
<213> Artificial Sequence

<220>
<223> Synthetically generated SiRNA

<400> 52
ugggcgcccu ucaguucuga guu                                    23

<210> 53
<211> 21
<212> RNA
<213> Artificial Sequence

<220>
<223> Synthetically generated SiRNA

<400> 53
gauaggagcc cagcuucgag u                                      21

<210> 54
<211> 23
```

```
<212> RNA
<213> Artificial Sequence

<220>
<223> Synthetically generated SiRNA

<400> 54
acucgaagcu gggcuccuau cuu                                        23

<210> 55
<211> 23
<212> DNA
<213> Mus musculus

<400> 55
aagctggccc tggacatgga gat                                        23

<210> 56
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> "Dual targeting" siRNAs

<221> misc_feature
<222> 20, 21
<223> n = dT= deoxythymidine

<400> 56
uaccagcacc caggugcugn n                                          21

<210> 57
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> "Dual targeting" siRNAs

<221> misc_feature
<222> 20, 21
<223> n = dT= deoxythymidine

<400> 57
ccgggcaucc ggacgaguun n                                          21

<210> 58
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Dual targeting  siRNA

<221> misc_feature
<222> 1, 2
<223> n = dT= deoxythymidine

<400> 58
nnaugguagu gggucgacga c                                          21
```

```
<210> 59
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> "Dual targeting" siRNAs

<221> misc_feature
<222> 1, 2
<223> n = dT= deoxythymidine

<400> 59
nnggcccguc gcccagcuca a                                                  21

<210> 60
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Pseudocomplementary, bifunctional siRNA

<221> misc_feature
<222> 5
<223> n = A* = 2-aminoadenine

<221> misc_feature
<222> 20, 21
<223> n = dT= deoxythymidine

<400> 60
uaccngcacc caggugcugn n                                                  21

<210> 61
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Pseudocomplementary, bifunctional siRNA

<221> misc_feature
<222> 16
<223> n = A* = 2-aminoadenine

<221> misc_feature
<222> 20, 21
<223> n = dT= deoxythymidine

<400> 61
ccgggcaucc ggacgnguun n                                                  21

<210> 62
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Pseudocomplementary, bifunctional siRNA
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<221> misc_feature
<222> 1, 2
<223> n = dT= deoxythymidine

<221> misc_feature
<222> 7
<223> n = 2-thiouracil

<400> 62
nnauggnagu gggucgacga c                                    21

<210> 63
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Pseudocomplementary, bifunctional siRNA

<221> misc_feature
<222> 1, 2
<223> n = dT= deoxythymidine

<221> misc_feature
<222> 18
<223> n = 2-thiouracil

<400> 63
nnggcccguc gcccagcnca a                                    21
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